NEW SERIES VOL. 24 NO. 94 APRIL 1960

ANNALS OF BOTANY

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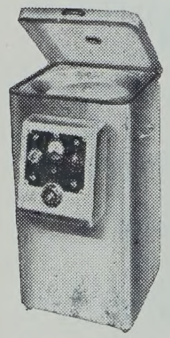

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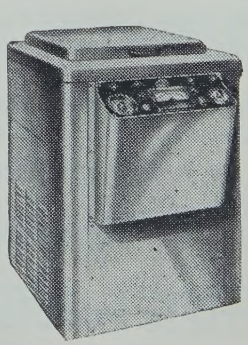

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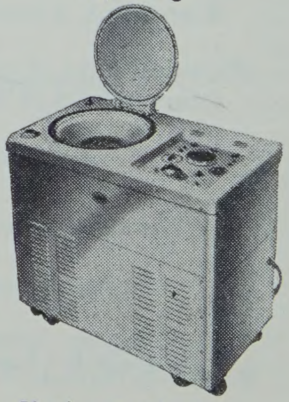

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The Effect of Annual and Bi-annual Pruning on the Seasonal Changes in Xylem Formation in the Grapevine

BY

ZVI BERNSTEIN[1] AND ABRAHAM FAHN

(*Department of Botany, Hebrew University, Jerusalem, Israel*)

With five Figures in the Text

ABSTRACT

Growth-ring development and seasonal changes in starch content were examined in annually as well as bi-annually pruned grapevines. Cambium was generally found to be active (in its normal season) from the middle of April to the end of July, with the main period of activity being May to June. As a result of an additional pruning in August a second period of cambial activity began in the middle of September and lasted until the middle of November, with the main activity occurring in October. In the 'normal' early summer season cambial activity was found to expand from the canes in a basipetal direction, whereas in the late summer season it recommenced simultaneously in the trunks and canes. In vines that had been pruned twice in the preceding year the early summer xylem increment was smaller than in those which had been pruned only once and the total increment of two growth-rings per year was similar to that of the one growth-ring in the latter, once pruned, plants.

INTRODUCTION

THE grapevine, a widely cultivated crop of the temperate zone, is also extensively grown in Israel. Here, vineyards are exposed to a wide range of climatic conditions. While winters are relatively cold in the hill regions, they are very short and mild in the Jordan Valley, where the mean minimum temperature of January and February is 14·4° C.

The vine, in contrast with other deciduous fruit trees and shrubs, has no need of a deep winter dormancy, which accounts for its succeeding also in these warmer areas (Chandler *et al.*, 1937). Under mild conditions, it is leafless for only two or three winter months and, with its fruit ripening in the early summer, retains its vegetative activity for a considerable period.

The extent of the growing season has induced Jordan Valley vinegrowers to prune their plants after the June–July summer harvest, so as to obtain an additional crop before the onset of the short winter (Stoler, 1954 *a*, *b*). There are thus two growth cycles within a year, a fact which has attracted the authors' attention, and has prompted them to investigate cambial activity and structural changes that occur in the xylem during the two respective growing seasons.

---

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[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

MATERIALS AND METHODS

The present investigation was carried out on vines grown as an irrigated crop in Kinnereth, which is situated in the Upper Jordan Valley at an altitude of about 120 metres below sea level. *Vitis vinifera* L. var. Chassela Doré, grown on rootstocks of *Vitis rupestris* Scheele var. du Lot was used.

The following methods were adopted:

1. Portions of outermost wood, whose length varied between 1 and 2 cm., and whose thickness and width was 0·5–1·0 cm., were removed from the trunk together with the bark. Two sets of plants were chosen, and samples were taken from alternate sets fortnightly—or at shorter intervals—throughout the two growing seasons. The wood portions were fixed in alcohol to which a little glycerin had been added. Permanent mounts of cross-sections stained with safranin and fast green were prepared from part of the material; other cross-sections were examined unstained and embedded in glycerin.
2. Pieces of canes were cut off at various time intervals and treated as above.
3. In order to determine cambial activity the strip method of Priestley *et al.* (1933) was adopted.
4. Cross-sections of canes and trunk portions removed at various time intervals were treated with I_2KI for starch determination.

PHENOLOGY OF THE EXAMINED PLANTS

Grapevine pruned in the usual way, i.e. once yearly in winter

The observations made on vines pruned annually to two buds during the winter months were as follows (Table 1).

TABLE 1

Phenological Observations on Grapevines Pruned Annually in Winter

	Date of			
Year	Bud burst	Beginning of flowering	Beginning of fruit ripening	Harvesting
1952	1 March	20 April	14 June	10 July
1953	18 March	30 April	20 June	12 July
1954	4 March	21 April	12 June	9 July

Bud burst occurred at the beginning or the middle of March, and the plants were in full leaf after 2 weeks. In the second half of April, the flower buds began to open. The fruit ripened and was ready for picking as table fruit, with a sugar content of 14–15 per cent., in the first half of July. Around the middle of June, the bark of the young shoots started turning brown. Leaf-fall began at the end of November and was completed by the middle of December.

Grapevines pruned twice yearly, i.e. in winter as well as in the middle of summer

Here observations were made on vines which were being pruned in the usual manner to two buds in winter—and were then pruned a second time at the end of August after the summer harvest. At that time all leaves were removed, and the current season's canes were shortened to eight buds each.

Only one or two of the uppermost of these buds developed into shoots, which were to carry the second crop. Shoot development began 8–12 days after pruning (Table 2). Eighteen to twenty days later the flowers began to open. Fruit ripened at the beginning of December or beginning of January. Leaf-fall started at the beginning of January, i.e. about a fortnight later than in plants which had not been summer-pruned.

TABLE 2

Phenological Observations on Grapevines Pruned Bi-annually, i.e. in Winter and Summer

	1952 Pruned		1953/4 Pruned	
Phenological state	28 January	22 August	28 January	23 August
Bud burst	1 March	1 September	16 March	5 September
Beginning of flowering	18 April	19 September	30 April	25 September
Beginning of fruit ripening	14 June	15 November	20 June	20 November
Harvesting	10 July	4 December	12 July	8 January

GROWTH-RING DEVELOPMENT

The secondary wood has previously been described in detail by Esau (1948*b*), and Penzig (1882). It comprises broad rays, very wide to very narrow vessels the members of which, as a rule, have simple perforation plates although scalariform perforations also occur. The fibres are mostly septate with a nucleus in each compartment. The wood parenchyma is paratracheal but scattered parenchyma cells also occur among the fibres.

The growth-rings are easily distinguished by the flattened fibres produced at the end of each season as well as by the tangential expansion of the rays on the border between adjacent rings.

There is no differentiation into heart- and sap-wood in the grapevine. The pattern of tylose distribution is the same in both inner and outer stem regions: when Indian ink was passed through the wood, its rate of penetration was found to be the same in inner and outer growth-rings.

The formation of the ordinary growth-ring (early summer)

In a specimen in which bud burst had been recorded on March 4, 1954, the first signs of cambial activity were evident in a sample taken on April 11 when 2–4 newly formed cell-layers with incompletely thickened walls were seen in the xylem. There were approximately 10 such cell layers in a sample taken 14 days later, on April 25. Cambial activity was at its highest in a sample of May 7. Also at that date there were the first signs of lignification on the earliest formed vessels. Lignification of the fibres around the vessels started subsequently and proceeded at a somewhat slower rate. Cambium remained active, although not fully, till July. Similar early summer growth-ring formation was recorded in other specimens, both in those which had only been winter-pruned in the previous year, and in others which had been pruned in winter as well as in the preceding summer.

The formation of the additional growth-ring in late summer

The examined specimens which had been pruned in August began forming new shoots at the beginning of September. Resumed cambial activity was first observed in samples of September 17, in which a small number of cell layers belonging to a new growth-ring could be seen. Highest cambial activity was recorded in samples taken on October 9. By October 27 the growth-ring was complete (in all specimens examined).

Structure and course of development were essentially the same in the early and the late summer growth-ring.

CAMBIAL ACTIVITY AND ITS EXPANSION PATTERN

The rhythm of cambial activity, and its circumferential expansion in the trunk

Although an examination of sections is the most reliable method for ascertaining rates of cambial activity, a picture comprising the entire stem circumference is not easily deduced from a study of sections. For this latter purpose the strip method was adopted (Priestley *et al.* 1933). At each sampling strips of bark were peeled from three or four different sides of the examined trunk at a height of 20 to 30 cm. from the ground. Sampling was performed 16 times during the course of the growing season on 10 plants at various time intervals each. At critical periods of cambial activity these intervals were as short as 4 days. The results are set out in Table 3.

TABLE 3

Rates of Cambial Activity in the Trunks of Grapevines Pruned Bi-annually (further explanations in text)

Date of examination (1953)	Number of plants showing				Total number of plants examined
	Partial cambial activity	Full cambial activity	Minimal cambial activity	No cambial activity	
22 April . . .	3	—	—	7	10
26 April . . .	2	8	—	—	10
8 May . . .	—	10	—	—	10
18 June . . .	—	10	—	—	10
24 June . . .	5	5	—	—	10
8 July . . .	3	—	3	1	7
17 July . . .	6	—	1	2	9
23 July . . .	4	—	2	3	9
5 August . .	—	—	2	8	10
22 August . .	—	—	—	10	10
13 September . .	6	—	—	4	10
22 September . .	8	2	—	—	10
2 October . .	2	8	—	—	10
25 October . .	1	9	—	—	10
6 Nobember . .	5	—	2	3	10
13 November . .	1	—	3	6	10

Cambial activity was defined as full activity, when all bark could be peeled off easily; as partial activity, when some of the strips could not be made to slip; as minimal activity, when part of the bark could be removed with difficulty and, while the remainder could not be removed at all. Cambium was defined as non-active, when none of the bark could be loosened.

As is evident from Table 3, the periods of cambial activity can be divided into three phases: a first phase leading from partial to full activity, a second phase of full activity, and a third phase in which full activity changes to dormancy. These phases are evident in both the early and the late summer seasons of cambial activity. On comparing the two seasons one finds that: (*a*) the entire period of cambial activity in the early summer is longer than it is in the late summer; (*b*) the first phase, partial to full activity, is very short in the early summer, when it lasts a week or less, while it has a duration of approximately 2½ weeks in the late summer; (*c*) the second, full activity phase, extends over 7 weeks in early summer, while it lasts only 4 weeks in late summer; (*d*) the third, full activity to inactivity phase, lasts 5 weeks in early summer, and only a week and a half in late summer.

Phenology and phases of cambial activity in the trunk

In early summer 2½ weeks passed from the opening of the first bud to the stage when bud burst was general throughout, whereas in late summer only 5 days passed between initial and general bud burst. The respective time interval between initial bud burst and the first phase of cambial activity was 5 weeks in early summer, and a week and a half in late summer (Text-fig. 1).

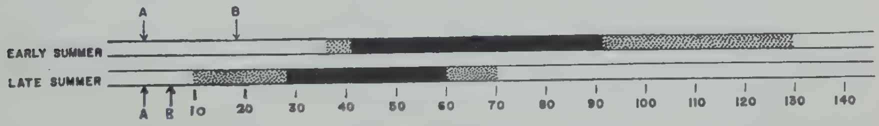

FIG. 1. Comparison between periods of cambial activity in the early and late summer seasons respectively. The numbers indicate days after beginning of bud burst, *A*. *B* represents full leaf stage. Full activity marked by solid black, partial activity by dotted areas.

The period that passed between general bud burst and the second phase of cambial activity, on the other hand, was the same in both seasons, i.e. about 3 weeks.

The rhythm of cambial activity in the cane as compared with that in the trunk

The course of cambial activity in the cane was studied with the aid of the strip method and checked several times by means of cross-sections: 10 to 20 canes were used for each examination.

In winter, pruned canes, as has already been mentioned, were cut back to two buds, and in summer to eight buds. The area beneath the topmost unfolding bud was taken as starting-point in a comparison of expansion patterns of cambial activity, irrespective of the total length of the cane.

When bark in 90 per cent. of the canes could be made to slip, *full cambial* *activity* was recorded, as against *partial cambial activity* where the percentage was lower. The phases of cambial activity in the canes as compared with the trunk are shown in Text-fig. 2. It is seen that in the early summer season the first phase in part of the canes starts 2 weeks earlier than it does in the trunk, whereas in the late summer season the phase of partial activity begins simultaneously in both trunk and canes. It can, therefore, be concluded that the slow basipetal expansion of cambial reactivation, which is evident in early summer, has no parallel in the late summer season. It may be that in the latter season patches of cambium become reactivated independently both in the canes and in the trunk.

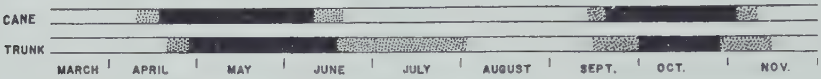

FIG. 2. Comparison between rhythms of cambial activity in cane and trunk.

The distribution pattern of cambial activity in the cane during the late summer season

Esau (1948*a*) investigated the pattern of cambial reactivation in the cane and came to the conclusion that cambial activity is reinitiated beneath the sprouting buds. The patches of active cambium expand downward, then fuse, and finally all cambium becomes active. Wray (1934) reports that, when dormant apple buds were stimulated into sprouting by summer pruning, the cambium situated immediately beneath these buds became reactivated, but active cambium remained confined to a limited area.

In the present investigation the expansion pattern of cambium, newly stimulated into activity by summer pruning of the canes, was examined. The following methods were used:

1. Expansion of reactivated cambium was traced by the strip method.
2. The rate of cambial activity was examined by means of transverse and longitudinal sections of the cane; in addition, successive measurements of the cane diameter were taken.

The pattern of expansion was found to be as follows:

During the first few days active cambium was restricted to small patches closely beneath the unfolding buds. These patches extended downward along the side on which they had been initiated. After approximately 10 days they had extended farther in a downward direction, as well as sideways, and now continued in two lobes which ran down the sides of the cane not occupied by buds (Text-fig. 3). At that stage, the earliest reactivated cambium, nearest the uppermost unfolding bud, had expanded around the whole circumference of the cane. The strips of cambium located on the sides of the cane occupied by dormant buds became reactivated last. It also happened that this part of the cambium did not recommence activity at all during the late summer season. Active cambium had expanded to the base of the 40–50 cm.-long cane

15 to 20 days after its reinitiation. In some cases, there was a downward expansion of the initial patches to no more than several centimetres. The extent of expansion seems to be correlated to the vigour of the side-shoot or shoots which develop on the cane.

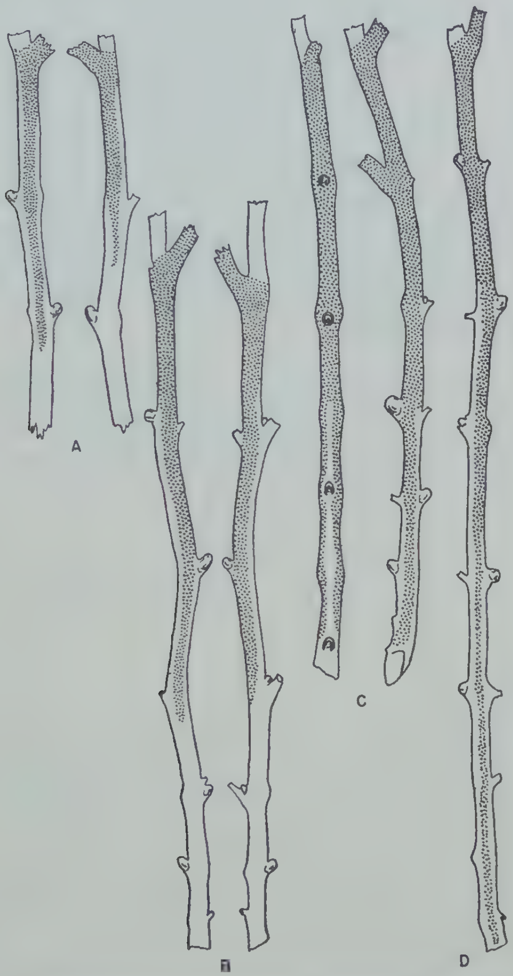

FIG. 3. Distribution pattern of cambial activity in the cane during the late summer season. Areas of active cambium marked by dotted area.

A—12 days after bud burst
B—16 ,, ,, ,,
C—20 ,, ,, ,,
D—24 ,, ,, ,,

As has been mentioned, cambial activity rates were studied by means of cane sections. Cross-sections were made at various levels of the cane, and longitudinal 1·5 cm. long sections in the nodal regions of dormant buds. A study of these, together with the data of successive measurements of the cane diameter, led to the following conclusions:

Xylem increments in the cane during the late summer season are uneven and vary at different levels and on different sides of the cane. The largest increment was found immediately beneath the sprouting bud. At that level newly formed xylem extended around the entire cane, but the increment was much larger beneath the bud than on the sides alternate with it. There was a gradual diminishment in a downward direction. At a level 3 to 4 internodes below the sprouting bud very small increments of xylem were found on the bud-bearing side of the cane, if at all. In cross-sections these increments appear as two crescents and account for an elliptical shape of the section.

No active cambium was found in the areas immediately surrounding dormant buds, even in regions where the cambium was active around the whole circumference of the internodes.

FLUCTUATIONS IN WIDTH OF GROWTH-RINGS

There appears to exist a certain irregularity in the trunks of the grapevine, as to the width of individual rings, and their degree of completion. An examination of whole trunk sections revealed that, especially in the early years of growth, there are rings which have not been fully completed and whose borders are only slightly demarcated. Later-formed growth-rings, especially in the stock, are often discontinuous and account for a furrowed outer appearance of the cane or trunk.

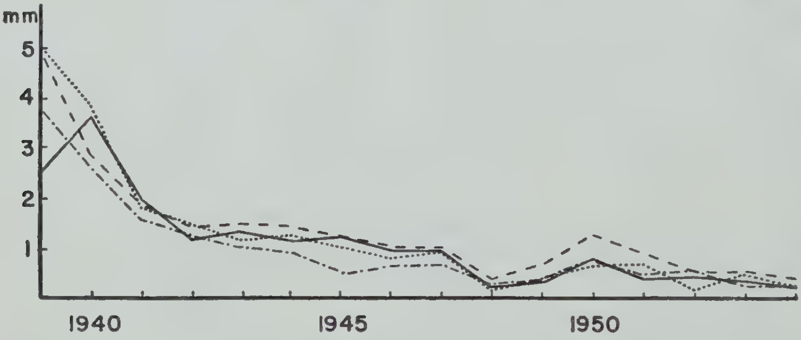

FIG. 4. Diagrams of growth-ring width of 4 trunks.

Changes in ring width were found to be correlated with age. To assess the width of individual growth-rings trunks were cut at a level of about 20 cm. from the ground, and averages were calculated from measurements on 4–8 radii of the cut surface. Ring width was observed to decrease with the age of the trunk, as shown in Text-fig. 4. In the early years rings are up to 5 mm. wide, whereas after the third ring there is a fluctuation in width between 0·1 mm. and 1·0 mm. Variation in ring width as influenced by bi-annual pruning and the production of an additional growth-ring was also examined. As it was impossible to cut entire specimens for this purpose, measuring was done on sectors of the trunk removed from one side of the plant only. From the results set out in Tables 4 and 5 it can be seen that: (i) the total width of two growth-rings produced as a result of bi-annual pruning did not generally exceed that of the single ring produced in the ordinary way; (ii) where two rings had been formed in one year, there was no significant difference in their respective widths, although in early summer the general vigour of vegetative growth had been more pronounced and the period of ring development had been longer than in late summer.

TABLE 4

Width in mm. of Annual Xylem Increment in the Trunk of Grapevines Pruned Annually

Serial number of plant	Annual increment					Total increment during 1951–5
	1951	1952	1953	1954	1955	
1 . . .	0·60	0·43	0·79	0·81	0·27	2·90
2 . . .	0·63	0·58	0·83	1·89	0·36	4·29
3 . . .	0·45	0·15	1·44	1·26	1·62	5·92
4 . . .	0·72	0·54	0·68	0·54	0·38	2·86
5 . . .	0·63	0·68	0·81	0·81	0·99	3·92
6 . . .	0·45	0·76	0·81	0·72	0·90	3·64
7 . . .	0·50	0·90	1·62	0·54	0·90	4·46
8 . . .	0·54	0·72	1·44	0·63	0·72	4·05
9 . . .	0·45	0·95	0·76	0·23	0·23	2·65
10 . . .	0·54	0·36	0·72	1·08	0·90	3·60

TABLE 5

Width in mm. of Seasonal Xylem Increment in the Trunk of Grapevines Pruned Bi-annually

Serial number of plant	Seasonal increment										Total seasonal increment during 1951–5		
	1951		1952		1953		1954		1955				
	Early summer	Late summer	Early summer	Late summer	Early summer	Late summer	Early summer	Late summer	Early summer	Late summer	Early summer seasons	Late summer seasons	Total increment during 1951–5
1	1·08	0·72	0·81	0·27	0·50	0·54	0·09	0·27	0·45	0·18	2·93	1·98	4·91
2	0·22	0·72	0·32	0·22	0·18	0·18	0·05	0·16	0·18	0·09	0·95	1·37	2·32
3	0·32	0·76	1·08	0·60	0·09	0·36	0·72	0·78	0·36	0·23	2·57	2·73	5·30
4	—	0·54	0·63	0·60	0·43	0·43	0·40	1·08	1·44	0·27	*2·90	*2·38	—
5	—	—	0·63	0·76	0·54	0·45	0·63	0·94	0·76	0·22	*2·56	*2·37	—
6	—	0·68	0·36	0·40	0·26	0·29	0·54	0·63	1·31	0·36	*3·47	*1·68	—
7	0·45	0·68	0·18	0·60	0·54	0·14	0·13	0·16	1·08	0·07	2·38	1·65	4·03
8	0·63	0·74	0·43	0·72	0·81	0·63	0·67	0·36	0·27	0·27	2·81	2·72	5·53
9	0·76	0·40	0·31	0·45	0·23	0·54	0·18	0·31	0·41	0·27	1·89	1·97	3·86
10	0·13	0·54	0·11	0·54	0·22	0·23	0·20	0·18	0·16	0·27	0·82	1·76	2·58

* Total of 4 years only.

An analysis of growth-rings was made in 5 trunks of 16-year-old plants. In one of the graphs in Text-fig. 5 the width of each growth-ring is expressed as percentage of the deviation from the average width of the two rings adjacent to it. A certain periodicity in width fluctuations is evident from that diagram. A ring which is narrower than its predecessor is followed by two rings of increasing width, while there is a decrease again in the fourth subsequently formed ring. The second graph in Text-fig. 5 shows parallel fluctuations in the total weight of canes produced by the respective plants in the same season.

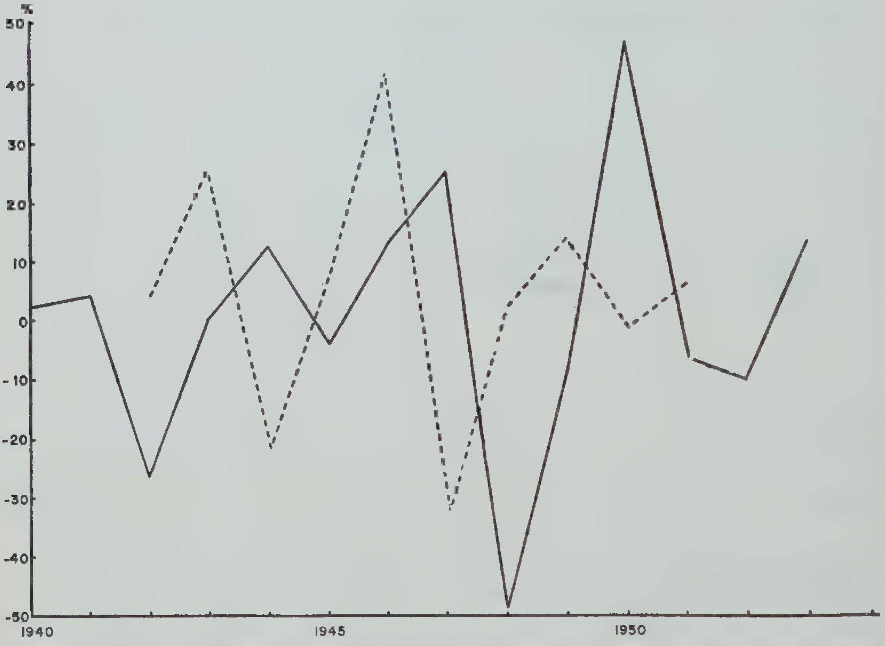

FIG. 5. Fluctuations in width of growth-rings (continuous line) and in total cane yield of same plants (interrupted line).

Here, too, the yield in weight of a particular season is expressed as percentage of the deviation from the average of the previous and of the subsequent season. Cane production as expressed in total weight can be taken to represent the main seasonal increment of vegetative growth. On comparing the two graphs it will be seen that each peak season of cane production is followed by a growth-ring of outstanding width, while a narrow growth-ring follows a season of little vegetative growth. On the other hand, no correlation between the yield in fruit and the width of the subsequently-formed ring was observed.

SEASONAL CHANGES IN STARCH CONTENT

Changes in starch content during the growing season were traced in cross-sections of newly formed shoots, mature canes, and trunks by treating them with I_2KI. In the new shoots, in the canes and trunks in which starch content had dropped to a minimum, starch grains were first found to reappear in the middle region of the wood rays. From here the accumulation of starch expanded toward the pith and in an outward direction toward the cambium. Simultaneously, starch grains also began appearing in the wood parenchyma and septate fibres. After the outward expansion had nearly reached the cambium zone via the wood rays, starch began forming in the phloem rays, again starting from the middle and expanding outward in two directions, as well as sideways into the phloem in a sequence similar to that described for the xylem. During the periods of fall in starch content, starch was seen to disappear completely from the phloem but not completely from the xylem. The seasonal changes of starch content in the trunk were found to coincide more or less with changes observed by Esau (1948*a*) in grapevine growing at Davis, California. By the time the new early-summer growth-ring approached the end of its differentiation, the phloem and the peripheral part of the xylem were completely depleted of starch. But before all the fibres of the new growth-ring were completely lignified, the starch content began to rise again and, spreading from the centre of the wood rays, starch reached the cambial zone at the end of August, i.e. about a month after cambial activity had ceased. The changes in starch content accompanying the differentiation of the second growth-ring proceed as in the first instance but starch increase at the end of ring differentiation takes place more rapidly.

THE FORMATION OF DORMANCY CALLUS

Our investigation of cambial activity throughout the year in grapevines grown in the Jordan Valley involved also a study of the formation of dormancy callus. The assumption of Esau's (1948*a*, page 268) that 'It is conceivable that in a milder climate than that of California, or in certain seasons, even the aerial plant parts could show incomplete dormancy or none at all' is not borne out by our observations. In the Jordan Valley with its relatively high winter temperatures, we found dormancy callus developing to an extent comparable with that described by Esau in California (Plate, Fig. 5). Callus was first found to be present at the end of October, about a month and a half before the leaves were shed. Leaf shedding here seems to be delayed by the absence of frost, yet callus formation proceeds at its appointed time. The amount of dormancy callus formed in 1953 was smaller than that formed in 1954, although no marked differences in climate were noted. In accordance with the findings of Esau we found very large amounts of dormancy callus in the canes, smaller amounts in the trunks, and no callus at all in the roots. The amount of dormancy callus found in the canes that had been formed after summer pruning equalled that found in canes that had developed in spring, i.e. in the usual season.

DISCUSSION

Grapevine plants, which were pruned in the middle of summer in addition to being pruned in winter, were found to have annually two growing periods. While the resumption of vegetative activity in spring follows a complete break of a long period of dormancy, the second renewal of vegetative activity in the late summer season occurs at a time when the plants are about to re-enter dormancy. The artificial stimulus of pruning, which arouses winter dormant buds into sprouting, undermines the process of entering dormancy. Vegetative activity is continued for only a short period, however, so that leaf-fall in summer-pruned plants begins only slightly later than that in ordinarily winter-pruned plants and overlaps with it. Differences in vegetative development, the production of canes with shorter internodes, and of shorter fruit clusters in the late summer season (Stoler, 1954 *a*, *b*), as well as differences in carbohydrate metabolism (Bernstein and Klein, 1957) indicate the dissimilar growth behaviour in the two respective seasons.

These differences in the two respective seasons are borne out by our observations of the course of cambial activity in the canes and trunk. While the expansion of renewed cambial activity in early summer is basipetal, starting from canes, reactivation in late summer begins more or less simultaneously in canes and trunk. In the latter it first appears in isolated patches, and becomes continuous only at a later stage. The cause for this simultaneous resumption of cambial activity in both the canes and trunk may lie in the fact that the trunk, the last part of the plant to have ceased activity previously, has not yet become fully dormant. Moreover, at the time of reactivation, growth substances which were present only a short time previously when the plant was in full leaf, and when cambium cells were still dividing, may still be available in sufficient amounts. Similar views with regard to evergreen trees were expressed by one of the present authors (Fahn, 1953, 1955) where the author ascribed simultaneous reactivation throughout the plant to the presence of sufficient amounts of growth-substances. According to Wareing (1951) cambial activity is resumed simultaneously in all organs, if a sufficient quantity of growth-subtances is available in the plant—providing adventitious buds have begun to sprout. The adventitious buds, which develop on the trunks of grapevines simultaneously with ordinary buds are prevented from acting as winter dormancy-breakers by the lack of sufficient amounts of growth-substances at the end of that relatively long dormancy. In the late summer seasons where such substances are available, adventitious buds do stimulate resumption of cambial activity in the trunk in the pattern mentioned. In canes pruned in the middle of summer, cambial reactivation, which spreads from the top downward, does not always expand around the entire circumference of the cane, or reach its base. This seems to be due to the preceding dormancy of the cambium, which began earlier and was more complete than that of the trunk. The extent of expansion corresponds with the relative vigour of sideshoots developing on the cane. The complete absence, or minimal activity, of cambium on sides of the cane-bearing dormant buds points to a possible influence exerted by these buds.

The shorter period of cambial activity in the late summer season is terminated by an abrupt change into the stage of entering complete dormancy. This latter stage coincides more or less with the normal period of leaf-fall. Although summer pruning induces renewed cambial activity in late summer and so changes the normal annual rhythm of radial growth, the factors controlling a normal course of development cause the second period of cambial activity to be much shorter and to cease more abruptly. In the following spring both normally and bi-annually pruned plants commence activity simultaneously.

The appearance of dormancy callus in the sieve tubes and its respective amounts in the various plant organs are indicators of the extent of dormancy. Esau (1948*a*) found dormancy callus most developed in the canes, less developed in the trunk, and altogether lacking in the roots. Our observations fit those of Esau's. Moreover, dormancy callus was found to be present in sideshoots that had developed in the late summer season, in quantities equal to or exceeding those found in the canes.

The considerable variations met with in the width of annual growth-rings are, in the authors' opinion, an outcome of the grapevine's fluctuations in vegetative growth and fruiting. The drastic pruning generally practised may often lead to a lack of balance between vegetative and reproductive growth. This determines the plentifulness or scarcity of food reserves stored in the woody parts of the plant, and especially in the roots which have a considerable influence on the vine's growing and fruiting capacity in the following year (cf. Winkler, 1945, 1954).

From the correlation between the weight of canes in a particular year and the width of the growth-ring that develops in the beginning of the summer following it, it appears that the extent of ring formation is dependent on the total photosynthetic output of the previous season. The fact that the growth-ring in the grapevine is formed early in the season and during a comparatively short period seems to support this view.

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Studies in the Physiology of Lichens

2. Absorption and Utilization of Some Simple Organic Nitrogen Compounds by *Peltigera polydactyla*

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With one Figure in the Text

ABSTRACT

Peltigera discs can accumulate relatively very large amounts of asparagine from solution by a process which involves the entry and accumulation of asparagine in the tissues as the complete molecule. Utilization of absorbed asparagine occurs at a relatively slow rate and involves the preliminary deamidation of it to ammonia. Asparagine absorption is completely inhibited by sodium fluoride, and partially inhibited by glucose. Absorption is accompanied by a release of ammonia in the medium and also by increases in respiration rates and a change in R.Q. None of the absorbed asparagine can be washed out of the tissues. Discs also show strong powers of absorption from solutions of glutamine, and glutamic and aspartic acids. There is more organic than inorganic nitrogen in solution in the liquids available to the lichen in its habitat.

The amounts of glucose and of phosphate which *Peltigera* and some other lichens can accumulate from solution are also very large considering the slow growth-rates of these plants. It is suggested that the possession of strong powers of absorption of substances from solution may be a general characteristic of lichens and plants of similar habitats: it may represent an adaptation to existence in barren conditions where the concentration of nutrients in solution is very low. Utilization of absorbed nutrients at a slow rate may be an adaptation to the need to survive long periods of starvation. The ecological implications of these suggestions are also discussed.

INTRODUCTION

THIS paper describes the results of experiments in which discs from the thallus of the lichen *Peltigera polydactyla* were incubated in solutions of some simple organic nitrogen compounds. Measurements were made of the amount of nitrogen absorbed by the discs and of the resultant changes in the composition of the discs and solutions. L-asparagine was the compound used in most of the experiments because preliminary work showed that absorption of nitrogen from asparagine solutions was much greater than from solutions of the other compounds (l-glutamine, l-glutamic acid, and l-aspartic acid); this phenomenon was therefore of especial interest since evidence given in the previous paper showed that it is unlikely that amides have an important quantitative role in the nitrogen metabolism of the discs. The results of the experiments to be described enable certain conclusions to be drawn not only about the nitrogen metabolism of *Peltigera* discs and their ability to absorb

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

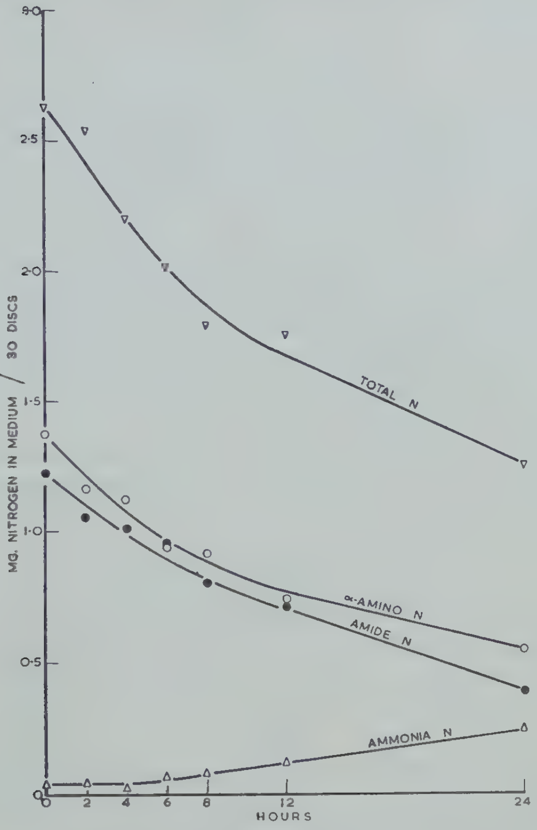

FIG. 1. Progress of changes in asparagine solutions during absorption by *Peltigera* discs at 20° C. Asparagine = 5 mM.

TABLE 1

Changes in Asparagine Solutions Resulting from Absorption by Peltigera *Discs. A Summary of the Results of 5 Experiments*

(In all experiments asparagine = 5 mM buffered to pH 5·7 in 10 mM phthalate. Absorption period = 24 hours at 20° C. Values for nitrogen in table = μg. N/100 mg. dry wt.)

Nitrogen Fraction	Experiment No.				
	21.2.57	20.3.57	15.5.57	24.6.57	3.9.57
Ammonia N released in medium . . .	394 ± 25	176 ± 14	96 ± 14	366 ± 17	96 ± 17
Loss of amide N .	1,306 ± 41	1,010 ± 24	1,004 ± 24	1,209 ± 29	784 ± 29
Loss of amino-N .	1,347 ± 47	1,157 ± 26	1,004 ± 26	1,211 ± 32	831 ± 32
Total loss of N from medium . . .	2,250 ± 78	1,971 ± 45	1,874 ± 55	2,105 ± 55	1,554 ± 78

substances from solutions, but also about possible aspects of the biology of lichens in nature.

METHODS AND MATERIALS

The methods of preparing samples and making extracts of discs, and of making quantitative estimates of various types of nitrogen compounds, respiration rates, and sugar absorption are the same as those described in the previous paper.

Absorption was studied by shaking samples of 30–50 discs in 20 or 25 ml. of aqueous solutions of the various nitrogen compounds for 24 hours in the dark at 20° C. Unless otherwise stated, the solutions were 5 mM and were buffered to pH 5·7 by M/100 potassium hydrogen phthalate. At the end of the absorption period estimates were made of the changes in the nitrogenous constituents of both the discs and the solutions.

The asparagine, glutamine, glutamic and aspartic acids used in the experiments were all commercial samples, and were all the l-isomers.

EXPERIMENTAL RESULTS

Absorption from Asparagine Solutions

The progress of absorption from asparagine solutions by *Peltigera* discs during a 24-hour experimental period is illustrated in Fig. 1. It can be seen both from this graph and from data in Table 1 that absorption has the following general characteristics: (*a*) it is of the relatively large order of 1,500–2,250 μg. N per 100 mg. dry wt./24 hours at 20° C.; (*b*) it is accompanied by a release of ammonia in the medium; (*c*) there is an almost parallel and equal loss of stable amide and amino-nitrogen from the medium during absorption. This latter characteristic suggests that asparagine enters the discs as the complete molecule, although it is possible that since ammonia is released in the medium during absorption, asparagine is first split into ammonia and aspartic acid before being absorbed. However, it is unlikely that this occurs to any

TABLE 2

Comparison of Absorption by Discs from Solutions of Ammonium Chloride, Aspartic Acid, Asparagine, and a Mixture of Ammonium Chloride and Aspartic Acid

(All solutions buffered to pH 5·7 by 10 mM phthalate)

Medium	Absorption (μg. N/100 mg. dry wt./24hrs)
5 mM ammonium chloride	226±22
5 mM aspartic acid	544±20
Equimolar mixture of 5 mM ammonium chloride and 5 mM aspartic acid	740±20
5 mM asparagine . .	1,970±45

great extent since data in Table 2 show that absorption from an equimolar mixture of 5 mM ammonium chloride and 5 mM aspartic acid is much smaller than absorption from 5 mM asparagine.

The view that asparagine enters the discs largely as the complete molecule during absorption is further supported by the results of analyses of discs after asparagine feeding which are included in Tables 3, 4, and 6. It can be seen that asparagine absorption results in a large increase in the soluble nitrogen of the discs equivalent to 87–94 per cent. of the loss of nitrogen from the medium. This increase is almost entirely in the fractions of ammonia, stable amide, and amino-nitrogen—the increase in stable amide nitrogen is equivalent to 60 per cent. of that lost from the medium, while the increase in amino-nitrogen is at the somewhat higher level of 73–78 per cent. of the loss from the medium. Evidence was given in the previous paper that amides do not have an important quantitative role in the nitrogen metabolism of the discs because the amount of amide nitrogen they initially contain is small and shows no change during starvation, ammonia feeding, or glucose feeding. Therefore the most credible explanation of the large increase in stable amide nitrogen in the discs during absorption from asparagine solutions is that the asparagine enters and accumulates in the discs as the complete molecule; the fact that there is also a large increase in amino-nitrogen during absorption would support this view.

If asparagine does accumulate in the discs to such a large extent during absorption, then this suggests that the rate of assimilation of asparagine is relatively low compared to its rate of entry. This is especially borne out by the data included in Table 3*b* which show the differences between discs analysed immediately after an absorption period and those analysed 72 hours afterwards. If the amount of stable amide nitrogen in the discs is taken to indicate the amount of asparagine they contain, then it can be seen that during the 72 hours the rate of disappearance of asparagine is slow, and at the end of it at least half of the asparagine which was originally absorbed still remains. Furthermore, the absence of significant changes in the amounts of either total soluble or insoluble nitrogen during this period suggests that the rate of synthesis of the more complex nitrogen compounds—if this is occurring at all—is very slow.

At least some of the asparagine nitrogen in the discs is converted to ammonia nitrogen. This is indicated by the increase in the ammonia nitrogen of the discs during asparagine feeding and also by the data in Table 3*b* showing that this increase continues during the 72 hours after the end of an absorption period. It seems very likely that deamidation of asparagine to ammonia occurs and in fact it could account for almost all the increase in ammonia nitrogen of the discs during and after asparagine feeding. The extent to which any deamination of asparagine to ammonia occurs is not clear, since changes in the amino-nitrogen of the discs may result from a number of causes besides the uptake and possible deamination of asparagine: evidence given in the previous paper showed that the amino-nitrogen of the discs increases both

TABLE 3*a*

Changes in the Nitrogen Fractions of Media and of Discs after Absorption from 5 mM Asparagine Solutions in the Presence and Absence of 16 mM Glucose

(Absorption period = 24 hours at 23° C., all solutions buffered to pH 5·8 by 10 mM phthalate. Values for nitrogen in table = μg. N/100 mg. dry wt.)

			−Glucose		+Glucose		
		Factor analysed	Control samples on water	Asparagine	Control samples on glucose	Asparagine	Differences significant at level $P = 0·05$
Fractions of soluble nitrogen	Ammonia N	Medium	—	+366	—	+265	34
		Discs	197	+629	182	+676	34
	Amide N	Medium	—	−1,209	—	−1,078	58
		Discs	43	+365	54	+127	70
	Amino-N	Medium	—	−1,211	—	−1,133	64
		Discs	204	+892	101	+870	52
	Unidentified N	Discs	454	−44	357	+41	86
Total Soluble N		Medium	—	−2,105	—	−1,906	110
		Discs	898	+1,842	694	+1,710	150
Insoluble N		Discs	2,792	+24	2,894	+260	190

TABLE 3*b*

Changes in the Nitrogen Fractions of Media and of Discs after Absorption from 5 mM Asparagine Solutions in the Presence and Absence of Glucose

(Conditions the same as in the experiment shown in Table 3*a*. Values for nitrogen in table = μg. N/100 mg. dry wt. unless otherwise stated)

			Glucose			Glucose		
		Factor analysed	Control samples on water	Asparagine	Samples 3 days on water after 24 hours on asparagine*	Control samples on Glucose	Asparagine	Differences signif. at level $P = 0·05$
Fractions of Soluble Nitrogen	Ammonia N	Medium	—	+119	+199	—	+11	34
		Discs	188	+232	+434	134	+486	34
	Amide N	Discs	9	+797	+504	12	+162	70
	Amino-N	Discs	383	+1,001	+845	282	+748	52
	Unidentified N	Discs	500	−129	+200	432	−116	86
Total soluble N		Medium	—	−2,026	+193	—	−1,386	110
		Discs	1,080	+1,900	+1,975	862	+1,258	150
Insoluble N		Discs	3,446	−192	−65	3,546	−51	190
Glucose absorbed (mg./100 mg. dry wt.)		Medium	—	—	—	37·4	30·7	2·4

*N.B.: Values for 'medium' in this column are results of the analysis of the water medium on which discs had been floating for 3 days after asparagine absorption, and do not include results of analysis of the original asparagine medium: these are in the adjacent column headed 'asparagine'.

during starvation and during assimilation of ammonia, and it is likely that it would decrease during synthesis of more complex nitrogen compounds.

It is probable that some of the ammonia formed in the tissues from the degradation of asparagine diffuses out into the medium—the data in Table 3*b* show that during 72 hours on water after an absorption period the discs release significant amounts of ammonia. This would provide an additional explanation to that suggested previously for the appearance of ammonia in asparagine solutions during absorption.

TABLE 4

Changes in Discs and in Medium after Absorption from Asparagine Solutions with and without Sodium Fluoride added to the Solutions

(Absorption period = 24 hours at 20° C., all solutions buffered to pH 5·7 with 10 mM phthalate. Values for nitrogen in table = μg. N/100 mg. dry wt.)

		Factor analysed	Control samples on water	5 mM Asparagine	5 mM Asparagine +0·025M NaF	Changes significant at level $P=0·05$
Fractions of soluble nitrogen	Ammonia N	Medium	—	+96	+96	34
		Discs	174	+220	−136	34
	Unstable Amide N	Medium	—	0	0	46
		Discs	6	+7	−4	51
	Stable amide N	Medium	—	−784	+30	58
		Discs	8	+580	−6	70
	Amino-N	Medium	—	−834	+376	64
		Discs	249	+642	−249	52
	Unidentified N	Medium	—	+32	+178	—
		Discs	473	−169	−310	86
Total soluble N		Medium	—	−1,554	+680	110
		Discs	910	+1,280	−705	150
Insoluble N		Discs	2,672	−2	+22	190

There are a number of lines of evidence which indicate that absorption of asparagine is an active process, apart from the fact that data in Table 3*b* show that none of the absorbed asparagine can be washed out of the discs. The data in Table 4 show that absorption is inhibited by 0·025M sodium fluoride—in fact, it can be seen that treatment with sodium fluoride results in the discs losing 75 per cent. of their soluble nitrogen in 24 hours, although there is no loss of insoluble nitrogen. All the fractions of soluble nitrogen which increase in the discs during absorption from asparagine solutions show decreases if sodium fluoride is added, and the solutions contain more nitrogen at the end of the absorption period than at the beginning.

Further evidence that absorption of asparagine is an active process is given by the fact that it is accompanied by increased rates of respiration. This is shown in Table 5 which, for the sake of comparison, also includes data on the respiratory increases associated with glucose uptake. It can be seen that addition of asparagine to the medium results in increased rates of oxygen uptake and carbon-dioxide output, and there is a rise in R.Q. The increases in respiration rates are not as great as those resulting from the addition of glucose, but if it is assumed that asparagine enters the discs as the complete molecule, then it can be seen that the molar rate of uptake of glucose is greater than that of asparagine, so that the respiratory increases per mole of asparagine absorbed are at least as great as those per mole of glucose absorbed.

TABLE 5

Respiratory Responses of Discs to the Addition of Glucose or Asparagine. Warburg Experiments in which Rates were Measured over 3 hours, at 20° C.

(All media buffered to pH 5·8 by 10 mM phthalate)

	Respiration rates (μl./hour/100 mg. dry wt.)			Absorption rates (μmole/hour/100 mg. dry wt.
	O_2 Uptake	CO_2 Output	R.Q.	
Basic respiration rates .	82	52	0·64	—
After addition of glucose .	+49	+106	1·22	6·8
After addition of asparagine	+38	+55	0·87	3·3

The Effect of Glucose on Absorption from Asparagine Solutions

It can be seen from data in Tables 3*a* and 3*b* that addition of glucose to asparagine solutions results in a decrease in absorption by the discs. Both the loss of nitrogen from the solutions and the gain in nitrogen by the discs are significantly less in the presence of glucose than in its absence. The extent of this inhibition by glucose is more accurately portrayed in Table 3*b*, since in the experiment shown in 3*a*, the absorption period was accidentally prolonged so that by the end of it virtually no nitrogen remained in the media lacking glucose.

These results are especially interesting since it is known that glucose stimulates the uptake of nitrogen from asparagine solutions by the fungi *Endomycopsis vernalis* (Kating, 1955) and *Neocosmospora vasinfecta* (Harley, unpublished). When either of these fungi is grown in asparagine media without glucose, the loss of amide nitrogen from the medium occurs at a greater rate than the loss of amino-nitrogen, and large amounts of ammonia are released into the medium. Addition of glucose has little effect on the loss of amide nitrogen, but the loss of amino-nitrogen is increased to a rate approaching that of the loss of amide nitrogen, and the release of ammonia into the medium is considerably reduced. Thus, in the presence of glucose, the changes in the nitrogen fractions in asparagine media during absorption by these fungi are similar to those that occur when *Peltigera* discs are incubated in asparagine solutions without glucose.

It could therefore be argued that *Peltigera* discs contain sufficient internal carbohydrate to render external addition of glucose superfluous. However, this seems unlikely, since evidence given in the previous paper showed that addition of glucose to ammonium chloride solutions causes a marked stimulation of ammonia uptake by the discs. Furthermore, the values for R.Q. given in Table 5 suggest that the internal level of carbohydrates available for respiration is low.

It was shown in the previous section that asparagine absorption by the discs is probably an active process. It would therefore be expected that the provision of additional respiratory substrate for the discs might result in a stimulation of absorption. The fact that glucose does not stimulate absorption indicates either that the glucose which enters the discs is not utilized as a respiratory substrate, or that there is some factor other than the supply of respiratory substrate which limits the rate of absorption. The existence of such a rate limiting factor is suggested by the results in Table 3 which show that not only is asparagine absorption depressed in the presence of glucose, but also glucose absorption is depressed in the presence of asparagine. It is unlikely that glucose and asparagine compete for a common uptake mechanism; it is more likely that there is a competition between two separate uptake mechanisms for some common factor which is in short supply such as high energy phosphate compounds.

Table 3 shows that the effect of glucose on absorption is not simply to retard the rate of entry of asparagine into the tissues. The increase in the amide nitrogen of the discs and the release of ammonia in the medium are both smaller than would be expected if this were the case; similarly, the increases in the ammonia and amino-nitrogen of the discs are both larger than would be expected. It is likely that glucose in some way promotes the deamidation of asparagine to ammonia in the discs—this would account for the relatively small accumulations of amide nitrogen and large accumulations of ammonia nitrogen. It is known from evidence given in the previous paper that glucose promotes the conversion of ammonia to amino-nitrogen in the discs and this would help to account for the relatively large accumulation of amino-nitrogen. The fact that much less ammonia is released in asparagine solutions when glucose is added can be explained if it is assumed: (*a*) that at least some of the ammonia released in the solutions has diffused out of the discs; and (*b*) that the suggestion made in the previous paper that glucose increases the ability of the discs to hold ammonia is true. The addition of glucose during asparagine absorption would enable the discs to hold more of the ammonia released by the degradation of asparagine, so that less would diffuse out into the medium.

Absorption from Solutions of Organic Nitrogen Compounds other than Asparagine

Table 6 illustrates the fact that absorption of nitrogen from solutions of glutamine, glutamic acid, and aspartic acid was of a smaller order than absorption from asparagine. Absorption of all these compounds was accompanied by a release of ammonia in the medium, and by significant increases in the amounts of ammonia and amino-nitrogen in the discs.

In the case of glutamine, the release of ammonia in the medium was relatively large. Also, no unstable amide nitrogen could be detected in the medium at the end of the absorption period although abundant amino-nitrogen remained. Within the discs the increase in ammonia nitrogen was again relatively large, but there was no significant increase in the amount of unstable amide nitrogen. This complete disappearance of the unstable amide nitrogen accompanied by the marked increases in ammonia, both in the medium and in the tissues, suggests that glutamine is much more easily deamidated by the discs than is asparagine and that at least some of this deamidation occurs at the surface of the tissues.

The fact that absorption from glutamic acid exceeds that from aspartic acid is in agreement with the results of Algeus (1950, 1951) on *Scenedesmus* and other algae, although from these results one cannot go so far as Algeus in concluding that glutamic acid occupies a more central position in nitrogen metabolisms than aspartic acid. It is probable that deamination of both these acids can occur since during their absorption there is an increase of ammonia in the discs and in the medium.

TABLE 6

Changes in the Nitrogen Fractions of Media and of Discs after Absorption from Solutions of Asparagine, Aspartic Acid, Glutamine, and Glutamic Acid

(Absorption period = 24 hours at 20° C.; all solutions buffered to pH 5·7 by 10 mM phthalate. Values for nitrogen in table = μg. N/100 mg. dry wt.)

		Factor analysed	Control samples on water	5 mM asparagine	10 mM aspartic acid	5 mM glutamine	10 mM glutamic acid	Changes significant at level $P=0{\cdot}05$
Fractions of soluble nitrogen	Ammonia N	Medium	—	+95	+74	+469	+79	34
		Discs	223	+202	+67	+322	+239	34
	Unstable amide N	Medium	—	—	—	524	—	46
		Discs	18	−16	−5	−18	−4	51
	Stable amide N	Medium	—	−1,004	—	—	—	58
		Discs	35	+669	−10	−10	+1	70
	Amino-N	Medium	—	−1,004	−490	−879	−1,042	64
		Discs	273	+784	+314	+492	+493	52
	Unidentified N	Medium	—	—	—	—	—	—
		Discs	425	+37	+93	+146	+78	86
Total Soluble N		Medium	—	−1,874	−457	−907	−927	110
		Discs	974	+1,686	+459	+932	+807	150
Insoluble N		Discs	2,748	+77	—	—	—	190

If glutamic and aspartic acids, like asparagine, enter the discs as the complete molecules during absorption, then it can be seen that although the amount of nitrogen absorbed from asparagine solutions is much greater than that absorbed from solutions of either of the other amino-acids, the molar rate of uptake of glutamic acid would in fact be similar to that of asparagine, while the rate of uptake of aspartic acid would be approximately half this value.

Nitrogen in the Habitat of Peltigera

Since discs of *Peltigera polydactyla* can readily absorb a number of simple organic nitrogen compounds, it was of interest to discover the relative amount of organic nitrogen available to it in solution in its habitat. The plants used in the experiments grew on the slopes of disused clay pits in amongst tussocks of *Agrostis* sp. and various mosses beneath young trees of *Betula pubescens.* A preliminary investigation was made of (*a*) the nitrogen content of water trickling down the slopes after a light rain shower; and (*b*) the water-soluble nitrogen content of the surface 5 mm. of soil on the slopes at the same time. The water trickling down the slopes was collected in conical flasks, filtered, slightly acidified, and evaporated down to a convenient volume *in vacuo* at 40° C.; the nitrogen content was then estimated by methods similar to those used for extracts of discs. For estimates of soil nitrogen, about 40 g. of soil was scraped from the surface in the vicinity of some *Peltigera* plants, shaken in distilled water for 10 minutes, filtered, and then treated in the same manner as the sample of surface water.

The results of the analysis showed that ammonia comprises only 12 per cent. of the total (Kjeldahl) nitrogen in both samples; amino-nitrogen comprises 6 per cent. of the total in the surface water, and 25 per cent. of the total in the surface soil. The relatively low percentage of ammonia in the surface water may seem surprising in view of the fact that it is usually the main nitrogenous constituent of rain water. However, Tamm (1953) has shown that rain coming down through tree crowns has a lower ammonia concentration than that coming down in the open; he also showed that water coming through tree crowns has a different mineral composition, and a much higher content of organic matter. Tamm attributes these differences to the fact that the rain will dissolve dust particles caught in the tree crown and also animal excrement. Similar considerations would presumably apply in the case of the *Peltigera* plants, since much of the rain reaching the plants would have passed not only through the canopy of young birch trees, but also through the tufts of grass and moss which partially shelter them.

It is thus evident that much of the nitrogen in the water with which *Peltigera* comes into contact in nature is in organic form, and it is possible that organic nitrogen may be a more important nitrogen source for this and other lichens and plants of similar habitat than has hitherto been appreciated. A study of the types of nitrogen compounds in the habitats of lichens and of the extent to which such compounds can be utilized should help to settle the existing controversy over the extent to which the distribution of so-called 'nitrophilous' and 'nitrophobous' lichens is in fact determined by nitrogen supply (e.g. review by Barkman, 1958).

DISCUSSION

The experiments described in this paper provide good evidence that when *Peltigera* discs are incubated in asparagine solutions, they absorb large amounts of asparagine by a process which involves the entry and accumulation of asparagine in the discs as the complete molecule. There is also good evidence that absorption is an active process since it is accompanied by increased rates of respiration and a change in R.Q.; it can be inhibited by sodium fluoride, and none of the absorbed asparagine can be washed out of the discs. The changes occurring in the asparagine solutions during absorption by the discs differ in two main respects from those observed in the case of the few free-living fungi in which this problem has been studied.

(1) Large amounts of ammonia are released in the medium by the fungi, but only small amounts in the case of the discs. It is therefore possible that the fungi possess an active surface asparaginase which is either not possessed by the lichen discs or, if it is possessed, is inhibited by experimental conditions such as the pH of the medium. However, it is also possible that at least some of the ammonia released during absorption is the result of diffusion out of the tissues, so that the relatively small amount released by the discs may be partly a reflection of their marked ability to hold internal ammonia, and partly of the fact that the discs may utilize asparagine at a slower rate than the fungi. (2) Glucose stimulates absorption by the fungi, but partially inhibits absorption by the discs. The explanation of this is obscure, but since there is a degree of competition between asparagine and glucose absorption by the discs, it is possible that the rate of uptake of these substances is limited by factors other than the supply of respiratory substrates in the case of *Peltigera* discs, but not in the case of the fungi.

Although there is no evidence as to the mechanism involved in the absorption of asparagine and other organic nitrogen compounds by *Peltigera* discs, it is interesting to compare some of the results described in this paper with those obtained by Birt and Hird (1956, 1958*a*, 1958*b*) in their study of amino-acid absorption by carrot slices. These authors found that carrot slices can actively absorb a wide range of amino-acids, and on the basis of competition studies they concluded that a common carrier system was involved in the absorption of all the acids. These studies also suggested that there was great variation in the affinity of the acids for the system: l-amino-acids have a greater affinity than the corresponding d-amino-acids, and acids with lipophilic side chains have a greater affinity than those with hydrophilic side chains. It is possible that similar considerations might apply in the case of *Peltigera* discs, since as with carrot slices aspartic acid is absorbed at a slower rate than asparagine and glutamic acid.

After asparagine is absorbed by the discs it is deamidated to ammonia, and this supports the view expressed in the previous paper that amides do not play an important quantitative role in the nitrogen metabolism of the discs. The rate of deamidation is slow, indicating that the rate of utilization of asparagine is relatively very low compared to its rate of entry during absorption. This does not necessarily imply that asparagine cannot be easily utilized by the discs, because such evidence as there is suggests that rates of nitrogen metabolism as a whole in the discs are low: in none of the experiments described in this or the previous paper was any significant increase in the insoluble nitrogen fraction of the discs detected, even though the amount of soluble nitrogen may have trebled as a result of absorption. Furthermore, the changes during starvation of the discs that were described in the previous paper occurred at a slower rate than those known to occur in other types of plants.

The results of the experiments described in this paper also provide a further illustration of the fact that *Peltigera* discs show strong powers of absorption from solutions of a wide variety of substances. In so far as it is possible to make valid comparisons, the rate of asparagine absorption per unit weight of lichen discs is approximately twice that recorded by Birt and Hird for carrot slices, and by Kating for *Endomycopsis vernalis*. Table 7 summarizes the rates of absorption that have been recorded in all types of experiment so far performed with *Peltigera* discs. It is reasonable to suppose that at least some other lichens besides *Peltigera polydactyla* possess strong powers of absorption, since data published by Harley and Smith (1956) showed that a number of species have high rates of glucose absorption, and since it is known that thallus tips of *Parmelia physodes* can absorb phosphate at a rate similar to *Peltigera* discs (Smith, unpublished).

TABLE 7

Summary of Rates of Absorption of Substances from Solutions by Peltigera *Discs*

(In all cases concentration of solution = 5 mM and pH maintained within the range 5·6–5·8 by 10 mM phthalate buffer)

Solution	Absorption (per 100 mg. dry wt./ 24 hours at 20° C.)
Glucose . . .	22 mg. glucose
Asparagine . . .	2·0 ,, nitrogen
Aspartic acid . .	0·5 ,, ,,
Glutamine . . .	0·9 ,, ,,
Glutamic acid . .	0·9 ,, ,,
Ammonia in presence of glucose . . .	0·6 ,, ,,
Potassium phosphate .	1·3 ,, phosphorus

Since lichens are characteristically such slow-growing plants, the rates of absorption indicated in Table 7 are remarkably high, even taking into consideration that these are values obtained under laboratory conditions. The material used in these experiments grew at a rate of approximately 4 cm. a year, and this is probably a relatively high value since published figures for the growth-rate of foliose lichens rarely exceed 2 cm. a year. On the other hand, the apparently slow rates of asparagine utilization and of nitrogen metabolism as a whole in *Peltigera* discs are in keeping with a slow growth-rate.

If *Peltigera* and other lichens possess strong powers of absorption under natural conditions, and if it proves to be generally the case that absorbed substances are utilized at a slow rate, then this would suggest certain features about the biology of lichens in nature. Lichens do not possess special absorbing organs, and absorption of nutrients probably occurs over the whole thallus surface from any liquids passing over it. In the typically barren habitats of lichens the concentration of nutrients in solution is probably very low, so that strong powers of absorption might represent a necessity for survival in such conditions. As pointed out in the previous paper, a slow rate of utilization of nutrients may be an adaptation to the need to survive long periods of starvation. It may also be the case that during occasional periods of abundance of nutrients in the habitat the absorptive powers enable a reserve of nutrients to be built up which can be used in periods of scarcity: the lichen might thus function as an absorbing organ in a manner similar to that postulated by Harley (1956) for mycorrhizal roots. Plants other than lichens which grow in barren habitats may well exhibit these physiological characteristics.

It is possible that the marked sensitivity of lichens and other epiphytes to atmospheric pollution may be due partly to the possession of highly efficient mechanisms for accumulating substances from very dilute solutions, and partly to the fact that these plants have to rely almost entirely for nutrients on substances carried down in the rainfall from above. A similar explanation could be advanced for the observation of Gorham (1959) that mosses and lichens show greater accumulation of radioactive substances in areas of fall-out than do angiosperms.

SUMMARY

1. *Peltigera* discs can absorb relatively large amounts of asparagine from solutions by a process which is accompanied by increased rates of respiration. It can be inhibited by sodium fluoride, and none of the absorbed asparagine can be washed out of the discs.

2. Asparagine absorption appears to involve the entry and accumulation of asparagine as the complete molecule within the discs. The absorbed asparagine is utilized at a rate which is relatively slow compared to its rate of entry, and it involves the preliminary deamidation of it to ammonia. Glucose partially inhibits asparagine absorption.

3. Absorption also occurs from solutions of glutamine, aspartic, and glutamic acids. It is shown that there is more organic than inorganic nitrogen in solutions in the liquids available to the plant in its habitat.

4. The significance of the fact that absorption from solutions of organic nitrogen compounds, like absorption from solutions of sugars and phosphate, is of a relatively large order for such a slow-growing plant is discussed in relation to the biology of lichens in nature.

ACKNOWLEDGEMENTS

This investigation was carried out during the tenure of the Browne Research Fellowship at The Queen's College, Oxford. I am indebted to Dr. J. L. Harley for much helpful advice and criticism.

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Studies in the Physiology of Lichens

3. Experiments with Dissected Discs of *Peltigera polydactyla*

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ABSTRACT

The medulla of *Peltigera* discs can be separated by dissection from the overlying region which comprises the algal layer and upper cortex. In most cases, dissection has no marked effect upon the isolated tissue zones, so that a comparison of them gives a good approximate indication of the relative behaviour of the corresponding regions of the intact thallus. The behaviour of the medulla, entirely fungal in composition, gives some indication of the physiology of the lichen fungus.

The algal region has a greater respiration rate and nitrogen content than the medulla, and it absorbs more glucose, asparagine, phosphate, and ammonia than the medulla. Compared to the medulla, the algal region absorbs more glucose than asparagine and this is attributed to a marked preference for glucose on the part of the algal cells. The medulla has a greater water content than the algal region, and may function as a water reservoir.

INTRODUCTION

HARLEY and Smith (1956) developed a technique for dissecting discs of *Peltigera polydactyla* in which the medulla was separated from the overlying algal and upper cortical layers. The two tissue zones so produced were called the medullary and algal zones, and when they were incubated separately on sucrose solutions it was found that the algal zone was much more active in absorption than the medullary zone. This difference in absorptive capacity did not appear to be the result of special effects of the dissection technique, so that it was concluded that the algal zone was the main site of sugar uptake in the undissected thallus.

It was shown in the preceding paper that *Peltigera* discs may have remarkably strong powers of active absorption from solutions not only of sugars, but of other substances as well, and it was pointed out that this may be a significant phenomenon in the ecology of this and probably other lichens. In the experiments to be described in this paper, absorption by dissected *Peltigera* discs from solutions of a number of substances was studied in an attempt to determine if the algal zone was generally the main site of absorption in the thallus. The experiments also yield some information on other aspects of the physiology of *Peltigera* discs, and the fact that the medullary zone is entirely fungal in composition enables some conclusions to be drawn about the physiology of the lichen fungus. The extent to which the manipulation and selection of material involved in the dissection technique affects the

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.

results of the experiments is discussed in some detail, not only to facilitate the interpretation of results, but also because the experiments illustrate the potential value of the technique in investigating aspects of the physiology of the discs other than absorption.

METHODS AND MATERIALS

Dissection. The technique of dissecting *Peltigera* discs has been described in an earlier paper (Harley and Smith, 1956). It involves the selection of particularly thick discs which are first cut into quadrants of approximately equal size, and then the medulla from each quadrant is separated by making an incision immediately beneath the algal layer. The separated medullae are termed 'medullary zones', and the remaining tissues, which comprise the upper cortex and algal layer, are termed 'algal zones'. In order to assess the effects due to the selection and manipulation of material in this technique, most experiments include comparisons between the separated tissue zones, undissected quadrants from 'dissectible' discs (these are termed 'control quarter discs'), and 'normal discs'. The sampling procedure adopted in the experiments was as follows. Discs for dissection were sampled at random from a population of 'dissectible' discs. The quadrants from each disc were assigned one to each of four samples—two samples for dissection, and two for the 'control quarter discs'; where two treatments were being compared, one from each of these pairs of samples was assigned to each treatment. The sampling of quadrants was subjective, and aimed at producing samples of equal dry weight.

Experimental procedure. The experimental procedure for studying absorption of ammonia, asparagine, and glucose was the same as that described for normal discs in the previous two papers. It should be pointed out that this procedure differs from that described by Harley and Smith for studying sucrose absorption in that the tissue zones were shaken in 20 ml. media in stoppered conical flasks instead of being floated on 5 ml. media in small Petri-dishes. Table 1 shows that absorption of glucose by both zones is increased by shaking them, but that the medullary zone shows a greater increase than the algal zone. The explanation of this may be that in the flotation technique the medullary zones tend to sink to the bottom of the dish,

TABLE 1

The Difference between Shaking and Floating Tissue Zones in Glucose Solutions

(Absorption period = 24 hours in the dark at 20° C.; glucose = 28 mM., buffered to pH 5·6 by 10 mM. phthalate)

Tissues	Glucose absorption (mg./100 mg. dry weight) Samples floated	Samples shaken
Algal zones . . .	23·2	23·8
Medullary zones . .	8·8	11·9

and consequently may suffer from lack of aeration. The algal zones, like the undissected discs, always remain buoyant.

Phosphate absorption. Phosphate absorption was studied by shaking samples of discs or tissue zones in 20 ml. of 5 mM. potassium phosphate solutions labelled with ^{32}P for periods of 10–24 hours in the dark at 20° C.; all phosphate solutions were buffered to pH 5·6 by M/100 phthalate buffer. At the end of the absorption period the material was washed three times in distilled water and dried at 75° C. for 24 hours. Each dried sample was then ground to a fine powder and a known weight of powder—usually of the order of 12 mg.—was spread evenly on a thin nickel disc; the activity of the material was then measured with an end-window Geiger counter. The coefficient of variation of this method of measuring phosphorus accumulation was found to be 5·5 per cent.—this value was based upon 12 samples of normal discs, and includes variation due to the material as well as error in analysis. When discs which had been allowed to accumulate phosphorus for 10 hours were shaken in distilled water for 3 days, no significant increase in the radioactivity of the distilled water could be detected, showing that the washing of discs after phosphorus accumulation was adequate and that phosphorus accumulated by the discs is not released back into the medium.

The methods of making extracts of samples, and of making quantitative estimates of various types of nitrogen compounds, respiration rates, and sugar absorption are the same as those described in the two previous papers.

EXPERIMENTAL RESULTS

Ammonia Absorption in the Presence and Absence of Glucose

It was shown in a previous paper that *Peltigera* discs can absorb ammonia from ammonium chloride solutions and that absorption is stimulated in the presence of glucose. Table 2 illustrates the results of an experiment in which the effect of glucose upon ammonia absorption by dissected discs was studied. It can be seen that both zones can absorb ammonia, and that absorption is stimulated by glucose in each case.

A comparison of the behaviour of the two zones in the absence of glucose is of little value because dissection has a very marked effect upon their powers of absorption: the combined absorption of the zones is only 39 per cent. of that of the control quarter discs. Therefore the fact that the algal zone is the more active zone in absorption does not necessarily indicate that this is the main site of absorption in the thallus. Such a marked effect of dissection was not found in experiments where the absorption of other substances was studied. The significance of the effect on ammonia absorption will be discussed in a later section of this paper.

In the presence of glucose, the effect of dissection on ammonia absorption is not so marked, so that a comparison of the behaviour of the zones will give some information about the physiology of the whole disc. It can be seen that while glucose stimulates absorption by both zones, the response of the algal

TABLE 2

The Absorption of Ammonia by Dissected Discs in the Presence and Absence of Glucose

(All media contained 5 mM. NH_4Cl in 10 mM. phthalate buffer at pH 5·8. Glucose, when added = 16 mM. Absorption period = 20·5 hours)

Tissue	Medium	Ammonia absorption (μg. N/100 mg. dry wt.)	Glucose absorption(mg. 100 mg. dry wt.)
Algal zones	−glucose	88	—
	+glucose	420	23·4
Medullary zones	−glucose	61	—
	+glucose	190	11·8
Sum of zones	−glucose	75	—
	+glucose	315	17·8
Control quarter discs	−glucose	197	—
	+glucose	477	21·5
Normal discs	−glucose	189	—
	+glucose	548	—

zone is much greater than that of the medullary zone. This is probably correlated with the greater absorption of glucose by the algal zone: the ratio $\frac{\text{algal zone}}{\text{medullary zone}}$ for glucose absorption is approximately 2·0, and for ammonia absorption in the presence of glucose, 2·2. It is therefore reasonable to assume that the algal zone is the main site of absorption in the thallus both of glucose and of ammonia in the presence of glucose.

Nitrogen Content and Asparagine Absorption

Measurements were made in two experiments of the nitrogen content of the tissue zones, the amounts of asparagine they can absorb, and the changes in nitrogen content resulting from asparagine absorption. There was no significant difference between the two experiments, and the mean values obtained are shown in Table 3.

The algal zone has the greater total nitrogen content and the ratio $\frac{\text{algal zone}}{\text{medullary zone}}$ is approximately 1·8. The proportion of soluble to insoluble nitrogen is similar in both zones. A comparison between the two zones of the distribution of soluble nitrogen fractions shows that the greater amount of soluble nitrogen in the algal zone is due mainly to the larger amount of the 'unidentified' fraction in that zone.

Table 3 shows that both zones can absorb asparagine, and the ratio $\frac{\text{algal zone}}{\text{medullary zone}}$ for absorption is approximately 1·2. Thus, although the algal zone is more active than the medullary zone in absorption, the difference in activity between them is not as great as in glucose absorption. In both zones

TABLE 3

Nitrogen Content of Dissected Discs before and after Asparagine Absorption

(All media contained 5 mM. asparagine at pH 5·8 in 10 mM. phthalate buffer; incubation period = 24 hours at 20° C. in the dark. Columns headed '+asparagine' show the differences from control values. The figures for significant differences in the bottom line refer to total values and not changes from control values. All results expressed as μg. N/100 mg. dry weight)

	Nitrogen in discs												Changes in asparagine media	
	Soluble nitrogen										Insoluble nitrogen			
	Ammonia N		Amide N		Amino-N		Unidentified N		Total soluble N					
Tissue	Start of expt.	+ asparagine	Start of expt.	+ asparagine	Start of expt.	+ asparagine	Start of expt.	+ asparagine	Start of expt.	+ asparagine	Start of expt.	+ asparagine	Ammonia released in medium	Total nitrogen loss from medium
Algal zones	90	+93	63	+686	188	+730	545	+124	886	+1,681	3,650	+115	124	1,738
Medullary zones	88	+120	38	+491	160	+462	248	+147	534	+1,220	1,990	+74	271	1,450
Sum of zones	89	+105	52	+601	173	+617	416	+137	730	+1,460	2,810	+102	192	1,611
Control quarter discs	110	+128	66	+608	212	+601	376	+184	764	+1,421	2,832	+150	86	1,590
Normal discs	167	+250	29	+551	322	+661	336	−42	854	+1,506	2,940	−73	135	1,740
Differences significant at level $P = 0·05$	±24	±34	±20	±70	±30	±52	±60	±86	±84	±150	±186	±190	±34	±110

asparagine absorption results in large increases in the amide and amino-nitrogen of the tissues, suggesting that asparagine accumulates as such in both zones during absorption. Comparisons of the combined values of the tissue zones with the control quarter discs show that the process of dissection has not affected asparagine absorption except that the release of ammonia in the medium is stimulated. Since it was suggested in the previous paper that at least some of the ammonia released in asparagine media results from diffusion out of the tissues, the fact that dissection stimulates the release of ammonia may be correlated with the fact that it appears to reduce the capacity of the tissues to hold ammonia.

Respiratory Responses to the Addition of Asparagine and Glucose

Table 4 illustrates the results of Warburg experiments in which measurements were made of the rates of absorption of glucose and asparagine by the tissue zones, and of the increases in rates of oxygen uptake associated with absorption. Since there is good evidence that both glucose and asparagine enter the tissues as the complete molecule during absorption, the absorption rates are expressed on a molar basis so that a more satisfactory comparison can be made between glucose and asparagine.

TABLE 4

Respiratory Responses of Dissected Discs to the Addition of Glucose and Asparagine

(Rates of glucose, asparagine, and oxygen uptake measured over 3 hours in Warburg flasks. Glucose and asparagine = 5 mM. in 10 mM. phthalate buffer at pH 5·7)

	Absorption (μ mole/100 mg. dry wt./hr.)		Oxygen uptake (μl. O_2/100 mg. dry wt./hour)		
Tissue	Glucose	Asparagine	Basic rate	After addition of glucose	After addition of asparagine
Algal zones . .	8·5	2·8	121	+61	+9
Medullary zones . .	5·0	4·4	70	+19	+13
Sum of zones . .	7·0	3·5	100	+43	+11
Control quarter discs .	6·5	3·3	64	+54	+36
Normal discs . .	—	—	78	+63	+42

The rates of oxygen uptake of the tissue zones on phthalate buffer fall into line with results previously published by Harley and Smith (1956); the algal zone has a higher rate than the medullary zone, and a comparison of the rate for the control quarter discs with the combined rates for the tissue zones shows that dissection stimulates oxygen uptake. It can be seen from Table 4 that addition of glucose or asparagine to the tissue zones results in increased rates of oxygen uptake in all cases; the increases are of a relatively small order except where glucose is added to the algal zone when the response is large.

The measurements of absorption rates provide direct evidence that compared to the medullary zone, the algal zone is more active in the absorption of glucose than of asparagine. Furthermore, the absorption of glucose by the algal zone is accompanied by a proportionately greater increase in oxygen uptake than occurs during the absorption of glucose by the medullary zone, or of asparagine by either zone: the ratio μl. extra O_2/μ mole absorbed is approximately 7 when glucose is added to the algal zone, but is only 2–3 in the other cases. The two zones evidently differ in their mechanism of glucose absorption, and the most reasonable explanation for this would be that the mechanism of the alga differs from that of the fungus. Similarly, the greater preference of the algal zone for glucose could be explained on the basis that relative to the fungus, the alga prefers glucose to asparagine. However, the possibility cannot be ruled out that the fungal hyphae may be in different physiological states in the two zones: they are certainly anatomically different since the hyphae of the algal layer are smaller and have thinner walls than those of the medulla.

The fact that the respiratory stimulation of the algal zone is greater per mg. sugar absorbed than that of the medullary zone is the reverse of the conclusions of Harley and Smith (1956). This contradiction is due to the fact that Harley and Smith did not make direct measurements of sugar uptake in the Warburg flasks; they assumed that these would be similar to values calculated from experiments in which dissected discs were floated on sugar solutions for 24 hours. In practice, this assumption is not true because relative to the algal zone, the medullary zone has a higher uptake in Warburg experiments for two reasons: firstly, it was shown earlier in this paper that medullary zones absorb more when they are shaken in solutions than when they are floated; and secondly, comparison of the data in Table 4 with the data in Tables 2 and 3 shows that relative to the algal zone, the medullary zone is somewhat more active in absorption when this is measured over the first 3 hours of an absorption period—as exemplified in Warburg experiments—than when it is measured over longer periods as in other experiments.

Phosphate Absorption

Peltigera discs can absorb relatively large amounts of phosphate from potassium phosphate solutions by a process which is 'active' because it is completely inhibited in an atmosphere of nitrogen or by DNP, and because it is accompanied by large increases in respiration rates (Smith, unpubl.). Since phosphate absorption can be measured by using ^{32}P as a label, it is possible to obtain an estimate of the effects of dissection upon phosphate absorption by comparing discs dissected before and after absorption. An estimate of these effects would also be of value in interpreting the results of experiments described in previous sections where discs were always dissected before absorption.

Table 5 illustrates the results of an experiment in which the distribution of absorbed phosphate between the tissue zones of discs dissected before and after absorption was measured. It can be seen that both zones absorb phosphate, and that the algal zone is more active in absorption than the medullary.

TABLE 5

Discs Dissected Before and After Absorption of Phosphate Labelled with ^{32}P

(Absorption period = 10·5 hours on 4·5 mM. KH_2PO_2 buffered to pH 5·6 by 10 mM. phthalate at 25° C.)

	Absorption of phosphate (μg. P/100 mg. dry wt.)	
Tissue	Discs dissected before absorption	Discs dissected after absorption
Algal zones . . .	557	789
Medullary zones . .	432	461
Sum of zones . . .	497	657
Undissected 'dissectible' discs	—	647

Dissection of discs before absorption results in a 24 per cent. reduction in the amount of phosphate absorbed, and it is evident that this is due largely to an effect on the algal zone: the medullary zone is only slightly affected by dissection. In most of the experiments described in this paper, dissection causes a reduction in the amounts absorbed by the fungal zones, so that if the reduction is due mainly to an effect on the algal layer, then this zone may be even more active in absorption than the results of the experiments suggest. However, it must be pointed out that there is an alternative explanation for the results shown in Table 4; it is possible that during an absorption period there is a significant movement of absorbed phosphate between the tissue zones of undissected discs, so that the amounts of phosphate the zones contain at the end of the absorption period do not give a true picture of the absorptive capacities of the zones.

In none of the experiments described so far has dissection resulted in a stimulation of absorption, and this is evidence that the medulla does not form a significant barrier to the diffusion of dissolved substances to the algal layer in undissected discs. Further evidence is provided by the results in Table 5 which suggest that the algal zone may absorb even more in undissected discs than in dissected discs. However, in nearly all experiments the concentration of solutions used has been of the order of 5 mM., and it remains to be seen if the medulla does act as a barrier in solutions of the much lower concentrations that can be expected in nature.

Water Absorption

Table 6 illustrates the results of an experiment in which the water-holding capacity of the two zones was measured by the following means. Tissue zones from freshly dissected discs were floated on water until they had become fully saturated; they were then lightly surface-dried with filter-paper and weighed. Repeated weighings showed that there was a 3 per cent. error in the fresh weight determinations. The method of surface drying was such that no water droplets were retained on the external surfaces of the hyphae in the medullary zone.

TABLE 6

The Saturated Water Content of Dissected Discs. Results each Represent the Mean of Values Obtained from 3 Samples of 50 Discs each

Tissue	Saturated water content (mg. H_2O/100 mg. dry wt.)
Algal zones . . .	305
Medullary zones . .	375
Sum of zones . .	340
Control quarter discs .	338
Normal discs . .	320

The results of this experiment show that the medullary zone has a greater water-holding capacity than the algal zone. This would be in agreement with the views of a number of authors (e.g. Hilitzer, 1927; Stocker, 1927; Goebel, 1926) that the mechanism of water absorption by lichens is mainly an imbibitional process, and that much of the water in a saturated lichen thallus is held in the walls of the hyphae. In *Peltigera polydactyla*, the walls of the hyphae in the medulla are much thicker than those of the algal layer; this would therefore explain the difference in the water-holding capacity of the two zones.

Effects of the Dissection Technique on the Behaviour of Tissue Zones

The main object of the experiments described in this paper is to gain information about the physiology of different regions of whole, undissected *Peltigera* discs. It is therefore important to assess the extent to which both the selection of material and the manipulations involved in the dissection technique affect the behaviour of the isolated tissue zones.

It is evident from the results described both in this paper and by Harley and Smith (1956) that the specially thick discs selected for dissection are not markedly different from normal discs. Such differences as do occur can nearly all be explained on the basis that dissectible discs contain proportionately more medulla and less algal layer than normal discs, and that the behaviour of the algal and medullary zones relative to one another which is shown in dissection experiments persists at least approximately in the undissected discs. However, reference to Table 3 will show that there are some differences in the distribution of fractions of soluble nitrogen which cannot be explained in this way: dissectible discs contain less ammonia and amino-nitrogen and more unidentified nitrogen than is expected. It is possible that this may be due to a difference in age since dissectible discs come from older parts of the thallus where the medulla is thicker.

The effects of the process of dissection on absorption are variable and may be summarized as follows. Asparagine absorption is not significantly affected, but absorption of sugars and phosphate may be reduced by as much as a quarter. There is evidence suggesting that the reduction in phosphate absorption is due mainly to an effect on the algal zone and a similar effect probably occurs in the absorption of sugars. Thus, the absence of an effect of dissection on asparagine absorption may be related to the fact that the algal zone is relatively less active in the absorption of this substance, and if it is less active in asparagine absorption because the algal cells are less active, then the effects of dissection on the absorption of sugars and phosphate may result mainly from effects on the algal cells. Although the algal cells are not touched in dissection, there are two possible ways in which they might be affected: firstly, the incision beneath the algal layer made in dissection passes through a region which contains air-spaces even in a saturated thallus, so that the rupture of them in dissection may affect the aeration of the algal layer; and secondly, dissection might interrupt the movement of some substance from the medulla to the algal layer.

It is unlikely that these considerations explain the marked reduction in ammonia absorption caused by dissection. It was suggested in a previous paper that the amount of ammonia absorbed may be governed not so much by the rate of the absorption mechanism as by the ability of the tissues to hold ammonia, such as by the formation of ammonium salts of organic acids. If this is the case, the effect of dissection on ammonia absorption would be due to the removal of the free organic acid radicals: this might occur either through their participation in the increased amount of respiration which results from dissection, or they might be involved in the initial stages of the regenerative growth that is known to result from wounding in several *Peltigera* sp. (e.g. Lindahl, 1953). It was also suggested in a previous paper that the effect of glucose on ammonia absorption was to increase the ability of the tissues to hold ammonia. This would explain why dissection has a much smaller effect on ammonia absorption when glucose is added to the medium.

Harley and Smith noted that dissection causes a stimulation of the production sugars in sucrose solutions, and this was explained on the basis of an increase in surface area since a surface enzyme was shown to be involved. A similar explanation could be put forward for the stimulation of ammonia production in asparagine solutions caused by dissection, but this phenomenon is more likely to be allied to a decreased ability of the discs to hold ammonia when dissected, since it was shown that at least some of the ammonia released in asparagine solutions results from diffusion out of the discs.

Finally, it has been shown that dissection stimulates respiration. This could be due to an increase in surface area or it could be a wounding effect. Since it can be calculated from data published by Harley and Smith that the R.Q. is not significantly changed by dissection, it is reasonable to assume that the stimulation in respiration rate does not involve a change in respiratory substrate.

It can be concluded from this analysis that the effects due to the selection and manipulation of material in dissection experiments are, in general, not excessive. There is no reason to suppose that the properties possessed by the isolated algal and medullary zones are not also possessed by the corresponding regions of the undissected thallus; it is also probable that the quantitative differences between these regions of the thallus are at least approximately the same as those between the isolated tissue zones. The relationship between the two regions of the undissected thallus which is suggested by the results of the dissection experiments is therefore summarized in Table 7. Included in this table are values for the ratio $\frac{\text{algal zone}}{\text{medullary zone}}$ which take into account the fact that in the normal thallus, the dry weight of the algal zone per unit area is greater than that of the medullary zone.

DISCUSSION

It was shown in the previous paper that *Peltigera* discs possess remarkably strong powers of absorption of substances from solution. The results of the experiments described in this paper have shown that: (*a*) the property of absorption is not confined to a particular region of the thallus since both tissue zones can absorb ammonia, asparagine, glucose, and phosphate; (*b*) during a 24-hour period, the algal zone absorbs more per unit weight than the medullary zone. The values for the ratio $\frac{\text{algal zone}}{\text{medullary zone}}$ in undissected discs which are given in Table 7 show that most of the substances absorbed by the discs in a 24-hour period enter the algal zone. The algal zone consists of the algal layer together with the upper cortex of fungal pseudoparenchyma; it is unlikely that this latter tissue is very active in absorption, so that most of the substances absorbed into the algal zone probably enter the algal layer. It is therefore reasonable to conclude that the main site of absorption of substances in the thallus of *Peltigera* is the algal layer.

There is no direct evidence of the parts played in absorption by the separate algal and fungal components of the algal layer. The behaviour of the medulla, which is entirely fungal in composition, shows that the fungus can absorb substances from solutions, but the fact that it absorbs less per unit weight than the algal zone does not necessarily mean that the algal cells are highly active in absorption, because the fungal hyphae of the algal layer may be more active than those of the medulla; it has already been pointed out that an anatomical difference exists between the two types of hyphae. Indirect evidence of the participation of the algal cells in absorption is provided by the results of the experiment which showed that the respiratory increases of the algal and medullary zones were similar during asparagine absorption, but during glucose absorption the increase of the algal zone was much greater per mole of glucose absorbed than that of the medullary zone; it was also shown that, relative to the medullary zone, the algal zone absorbed more glucose than asparagine. This suggests not only that the algal cells absorb glucose, but also that compared to the fungal hyphae they are much more active in the absorption of glucose than of asparagine—if indeed they do absorb asparagine. That the algae should show a preference for glucose would not be altogether surprising since so many green tissues such as leaf discs show a marked ability to absorb sugars, and it is known that some

TABLE 7

The Physiology of the Different Regions of the Thallus of Peltigera polydactyla *as Suggested by the Results of Dissection Experiments*

	Mean thickness in normal discs	Dry wt. mg. per 'normal' disc	Nitrogen content (mg. N/100 mg. dry wt.)	Saturated water content (mg. H_2O/100 mg. dry wt.)	Oxygen uptake (µl. O_2/100 mg. dry wt./hr.)	Absorption (per 100 mg. dry wt./24 hrs. at 20° C.)				
						Glucose (as mg. glucose)	Asparagine (as mg. N)	Phosphate (as mg. P)	Ammonia (as µg. N)	
									−Glucose	+Glucose
Algal zone	Upper cortex = 30 µ Algal layer = 50 µ	1·4	4·5	305	121	23·4	1·7	1·11	88	420
Medullary zone	400 µ	0·9	2·5	375	70	11·8	1·4	0·86	61	190
Ratio of algal/medullary in normal discs	0·2	1·6	2·8	1·3	2·7	3·1	1·9	2·2	2·2	3·4
Per cent. effect due to dissection	—	—	—	0	+56%	−17%	0	−23%	−67%	−34%

free-living terrestrial algae can show excellent growth on sugar media in the dark.

The greater absorptive capacity of the algal zone is paralleled by its greater nitrogen content and its greater rate of respiration. It is interesting in this respect to note that Sosa-Bourdouil (1944) found that the algal region of *Usnea barbata* contains more nitrogen and has a much greater enzymic activity than the central axis of this lichen. It is not surprising that the algal zone should appear to be the region of greatest metabolic activity in these lichens since this is the region where contact between the symbionts occurs. In the majority of lichens the relatively thin algal zone is underlain by a relatively thick layer composed entirely of fungal hyphae, forming a tissue such as the medulla in *Peltigera* or the central axis in *Usnea*. One of the particular functions this tissue may have in many lichens is that of being skeletal. Another possible function is suggested by the results of the experiment described in this paper which showed that the medulla of *Peltigera* discs has a greater water-holding capacity than the algal zone. *Peltigera polydactyla*—like other lichens—has no special features for conserving water: Smyth (1934), for example, has shown that the rates of water uptake and loss by thalli of *P. polydactyla* are similar to those of an agar gel. Furthermore, Smyth showed that the rates of respiration and photosynthesis are linearly related to the water content of the thallus—this relationship has also been demonstrated by a number of authors for many other species of lichens (e.g. Stocker, 1927). Since the algal zone of *Peltigera* is a region of high metabolic activity, and since the degree of activity depends upon its water content, the fact that the medulla has a high water content suggests that it might function as a water reservoir for the algal zone.

There is good evidence that the absorption of sugars, asparagine, and phosphate by *Peltigera* discs is an 'active' process, and is thus dependent on an adequate supply of respiratory substrate. On the 'classical' theory of lichen symbiosis, these would be supplied by the photosynthetic activity of the algae. The fact that the medulla shows such a relatively strong capacity for absorption suggests either that it is efficiently supplied with respiratory substrate by the algae, or that it can respire organic substances absorbed from the moisture in its habitat. In fact, the marked capacity of whole *Peltigera* discs for active absorption in the dark which has been consistently shown in these experiments suggests that the material is not in any state of starvation when it is collected from its habitat: since it grows in shaded places, it must either have a very efficient photosynthetic mechanism, or it must be able to exist partly as a saprophyte.

SUMMARY

1. *Peltigera* discs can be separated by dissection into a 'medullary zone' comprising the fungal medulla, and an 'algal zone' comprising the algal layer and upper cortex.

2. Experiments are described in which the behaviour of the two tissue

zones is compared with respect to nitrogen content, water content, respiration rate, and absorption rates of glucose, ammonia, asparagine, and phosphate. Since in most cases dissection has no marked effect on the tissue zones, their behaviour is taken as an approximate indication of the relative behaviour of the corresponding regions of the whole, undissected thallus.

3. It is concluded that the algal layer is the most active region of the thallus in metabolism: it has the greater nitrogen content, respiration rates, and rates of absorption. It is particularly active in glucose absorption. The medulla has the greater water content, and may function partly as a water reservoir for the algal layer.

ACKNOWLEDGEMENTS

This investigation was carried out during the tenure of the Browne Research Fellowship at The Queen's College, Oxford. I am indebted to Dr. J. L. Harley for much helpful advice and criticism.

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The Effect of Artificial Wind on the Growth-rate of Plants in Water Culture

BY

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With Plate and three Figures in the Text

ABSTRACT

Young plants of *Brassica napus* (rape), *Hordeum vulgare*, and *Pisum sativum* growing in water culture have been exposed to four continuous wind speeds for from 4 to 5 days. The plants were exposed to natural daylight, humidity, and temperature in a wind tunnel in which only air movement was controlled. The wind speeds found among the plants in the four sections of this tunnel were approximately 0·3, 0·7, 1·7, and 4·0 m./sec. The results showed no significant change in relative growth-rate or net assimilation rate with wind speed.

Previously published results, obtained with plants in soil or sand culture, have differed from those in the present experiments in showing a fall in the amount of growth as wind speed increased. It is suggested that the reduction in growth found in these previous experiments was caused by partial drying out of the plants, whilst in the present experiments water-supply to the roots was abundant and this effect was considerably reduced.

INTRODUCTION

IN windy places wind apparently reduces growth. In confirmation of this it is found that the erection of a wind break will increase the amount of growth. However the wind break affects the microclimate and carbon-dioxide concentration near the leaves as well as the wind speed. Consequently for the elucidation of the effect of wind on plant growth we must turn initially to laboratory experiments where similar air is blown over plants at different speeds.

Bernbeck (1920, 1924), Finnell (1928), Martin and Clements (1935), Wrenger (1935–6), Rao (1938), Satoo (1948), and Whitehead (1957) have raised plants in calm air and examined them after a further period of growth in calm air or in wind speeds of between 1·5 and 27 m./sec. Their results, usually obtained with plants growing in soil and placed in front of a fan, show that the amount of growth is reduced as wind speed is increased. Generally their plants looked gnarled, final dry weight and the area of each leaf were reduced, and water loss (expressed on a leaf–area basis) increased progressively as wind speed increased. These laboratory experiments have confirmed under more controlled conditions the reduction in the amount of growth observed in windy places, but the wind speeds used have been high. Recorded measurements among herbaceous plants in the field

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[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

suggest that wind speed seldom exceeds 1 m./sec. (Warren Wilson and Wadsworth, 1958).

At very low speeds of air movement, experiments indicate that increase in the speed of air movement increases assimilation rate. It has been shown that the results of Deneke (1931) for whole plants and for cut shoots indicate a linear increase in assimilation rate with wind speed up to the highest wind speed examined of 0·9 m./sec. (Decker, 1947; Warren Wilson and Wadsworth, 1958). The results of Heinicke and Hoffman (1933), who used leaf chambers and low speeds of air movement, also show assimilation rate to increase with wind speed. Wadsworth (1959) has reported net assimilation rate to increase up to a maximum at around 0·3 m./sec. Haas (1939) shows results for a long-term experiment which suggest that a wind speed of about 0·4 m./sec increased final fresh-weights relative to calm air, but his results are of doubtful significance.

Thus from previous work it seems clear that there is an optimum wind speed for plant growth. This optimum speed varies. It is above 0·9 m./sec. under the conditions used by Deneke (1931) and around 0·3 m./sec. under the conditions used by Wadsworth (1959).

In most of the previous work with whole plants the amount of growth and not the rate of growth has been measured. The magnitude of differences in the amount of growth is affected by the duration of growth and the initial dry weight. These factors may affect growth-rates little. The distinction possible when calculating growth-rates between net assimilation rate and relative growth-rate enables the effect on the plant to be more fully examined. In this paper I report continuation of the growth analysis of plants grown in wind first reported by Wadsworth (1959). The plants have been grown in water culture so that their roots in all wind speeds should be equally supplied with water all the time; thus the effect of wind might be divorced from differences in water-supply to the roots caused by the soil or sand around the roots drying out in times of rapid evaporation. The plants used were rape (*Brassica napus* L.), barley (*Hordeum vulgare* L.), and pea (*Pisum sativum* L.) in four wind speeds of similar magnitude to those found in the field. My results have enabled me to discuss the nature of the factors which cause reduction in growth-rate at these normal wind speeds.

Apparatus and Methods

The Wind Tunnel

In these experiments I have grown plants at four wind speeds simultaneously. These wind speeds were produced in four sections, arranged in series, of a glass-sided wind tunnel placed on a roof where it is somewhat protected from natural winds. I will call the sections of this tunnel 1, 2, 4, and 8, which numbers represent the ratio of the wind speeds attained. Attempts to grow plants in still air were abandoned owing to the difficulties in maintaining conditions of temperature and humidity comparable to those in the tunnel.

In this tunnel a 30·5 cm. diameter aerofoil fan draws air from the largest section (1) to the smallest (8). The working sections are not rigidly connected to the section containing the fan and show little vibration. Each section is square in cross-section and has half the cross-sectional area of the preceding section. The narrowest section is 21·5 cm. and the widest 61 cm. across. Each working section has parallel sides and is joined to the next section by a contraction of 21° total angle (Fig. 1). The inside of the tunnel is smooth, all transverse members running outside the tunnel. On the contractions and on the top and one side of the working sections the glass sides are sealed with putty. The glass may be removed from the other side of the working sections where the glass is held by spring clips on to sealing strips of foam plastic. The floor of the tunnel is of hardboard with holes cut in it for the plant containers. These containers are enclosed in a wooden box underneath the working section and are supported so that the lid is flush with the floor of the tunnel. One stand of plants about 40 cm. long may be grown in sections 1 and 2 and two stands of this size in the narrower sections (4 and 8).

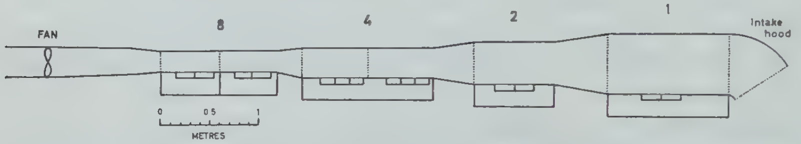

FIG. 1. Vertical longitudinal section of the wind tunnel. The fan on the left draws air through the tunnel from the right to the left through sections 1 to 8. The position of the tins in which the plants were grown (small rectangles), of the wooden boxes beneath each section which contain these tins (large rectangles), and of the screens across the tunnel (dotted lines) are indicated.

Smoke in the air stream revealed little turbulence. Disturbance of the air flow caused by plants in one section of the tunnel is removed by mesh screens at the beginning of each contraction and between the two stands of plants in sections 4 and 8. Nylon mesh screens of about 42 strands to the centimetre are placed over the beginning and end of the curved intake hood to remove most of the particles likely to block these mesh screens and to reduce the entry of wind eddies from outside the tunnel.

The tunnel is orientated in a north-south direction. The plants in the tunnel are subjected to the climatic variations of light, temperature, and humidity; only air speed is controlled. The structure and position of the tunnel cause reduction of the light inside and variation in light intensity between the sections. The light is somewhat reduced in all sections, section 4 is the brightest at 80 per cent. of full daylight and section 1 the darkest at 77 per cent. full daylight. The mesh screens shade some of the plants for a short time at mid-day. Changes in temperature and humidity of the air during the 5 seconds it takes to traverse the tunnel are small. On dull days and at night thermocouple measurements show no measurable change in the temperature or humidity of the air along the tunnel (< 0·03° C., < 0·1 per cent. R.H.). On bright days differences of 0·2° C. (maximum measured

0·3° C.) and 1 to 2 per cent. relative humidity (maximum measured 4 per cent. at 50 per cent. R.H.) were found.

Wind Speed Measurements

Measurements of air speed have been carried out using a shielded hot-wire anemometer of the type described by Simmons (1949). This instrument is very suitable for measurement of wind speed among vegetation since: (i) it can be made small, (ii) it retains its calibration for a long period, (iii) it may be read at a distance, (iv) it is not very sensitive to changes in the direction of air movement, (v) it is insensitive to changes in temperature and pressure, and (vi) it may be used close to solid bodies. The instrument is extremely sensitive to low wind speeds but less so to high wind speeds. It will distinguish between 5·0 and 5·1 m./sec. when reading on a scale 16 cm. long. Apparent sensitivity may be increased with special scales.

The sensing head of this instrument is composed of a heated twin-bore silica tube supported by a heating wire which passes through one bore. The amount that this tube is cooled by wind is measured with a thermocouple in the second bore. In the apparatus used the silica tube is 1 cm. long, and an average of 0·10 cm. in diameter; the heating wire is supported from two nichrome wires 0·12 cm. in diameter. The axis of the silica tube is at 45° to the axis of the handle. A constant current maintains the silica tube at a temperature of about 150° C. in still air. This head has been calibrated over a range from 0·03 to 5 m./sec. using a whirling arm (Cowdrey, 1950) at the low speeds and by comparison with a vane anemometer in the tunnel at higher wind speeds. No detectable variation in the calibration was found over the period the anemometer was in use.

In the empty tunnel, with the floor completed over the position of the plant stands, it has been found that the wind speed at the outlet of the contractions is highest near the sides and decreases towards the centre. Observations with smoke indicate that no eddying is initiated here. A boundary layer of more slowly moving air is formed quickly after each contraction. The main decrease in wind speed towards the tunnel floor, in the position of the stands, takes place in the first 3 cm. above the floor in sections 8 and 4, the first 2 cm. in section 2, and the first 1·5 cm. in section 1.

With plants in the tunnel the floor at the position of the stands is effectively raised and made uneven but only eddies of small size can be seen in smoke in the air stream. Where there are two stands in a given section air-speed measurements among the plants show that they experience similar wind speeds. Wind speed has been measured in the middle and to one side of the front and of the back of the stand, and in the centre of the stand. Where the leaf region was over 2 cm. thick wind speeds were measured at two heights. At each position measurements were made in a more exposed (between the rows of plants) and a less exposed (between the plants in a row) position. Measurements were made at the beginning and end of two experiments for rape and barley and of one experiment for pea. The plants were approximately the same size in each of the experiments on each species. The measurements showed great variation in wind speed, the extreme observed values overlapping markedly those of the neighbouring sections. Because of this large variation and the similarity between the two experiments with one species the mean of all the wind speeds measured for each species in each section was calculated. This value is given for each species in the table below together with the spread of two-thirds of the observations above or below the mean (in brackets).

	Wind speeds (m./sec) measured amongst the plants in the four sections of the tunnel			
	1	2	4	8
Rape	0·33 (0·2)	0·63 (0·3)	1·5 (0·8)	3·5 (1·4)
Barley	0·45 (0·2)	0·87 (0·3)	2·2 (0·7)	5·1 (1·4)
Pea	0·25 (0·2)	0·61 (0·3)	1·4 (0·7)	3·3 (1·9)

It will be seen that wind speed rather more than doubled between sections and that the wind speeds found among the plants of rape and pea were similar, whilst barley was exposed to rather higher wind speed because the stand was more open.

Growth of the Plants

Plants were raised from seed in sand culture out of doors. To obtain uniformity of growth the seeds were planted in a regular arrangement in sand. Soon after the seedlings appeared, plants of the middle size were transferred to water culture in a position close to the tunnel out of doors but protected from the rain. Each plant was individually supported over the nutrient solution in non-absorbent cotton-wool in a 0·7 cm. diameter hole in a halved 2·5 cm. cork shive. These shives were located 3·6 cm. apart in a 4 times 5 block on the 19·6 by 14·7 cm. lids of the water-culture tins. The tins and lids were painted inside with three coats of 'Bituros Solution' pure bitumen paint and on the outside with aluminium paint. The tins were 5·5 cm. deep and had two holes in the bottom. Through one of these air was bubbled and the other led to a constant level device. The water level in the tins was normally kept constant to within ±0·1 cm. by means of a constant drip feed to this device. The water level was maintained 1·9 cm. below the lid (1·0 cm. below the bottom of the shive). Each tin held one litre of nutrient solution for the 20 small plants.

Five hundred and twenty plants (26 lids) were transferred to water culture initially. They were grown in a nutrient solution containing macronutrients in the amounts used by Hoagland and Snyder (1933). The solution was made up with distilled water and a solution of five trace elements added. Iron was added in 1 per cent. ferric citrate solution at the rate of 1 ml. of solution per tin per day for rape and 1 ml. every other day for the other species. Healthy growth of all the species was maintained. After 3 to 4 days' growth in water culture the plants were transferred to the tunnel. Before transference the largest and smallest plants were removed leaving 400 plants (20 lids) for transference to the tunnel and up to 80 plants (4 lids) on which initial leaf area and dry weight were determined. The lids were selected so that in each section of the tunnel and in the initial sample allowance was made for possible differences in growth caused by differences in the position of the lids before transference.

The lids only were moved the short distance to the tunnel and placed on prepared culture tins already in position in the tunnel. The fan was then switched on. The position of the tins in the section is shown in Fig. 1. The stands are about 40 cm. long and occupy two-thirds of the tunnel width. The total number of plants in each section was 120 (6 tins) in sections 1 and 4, and 80 (4 tins) in sections 2 and 8.

The lids supporting the plants were turned around and changed in position in each section each evening. Plants at the back of the stand one day were thus at the front next day. In this way the effect of differences in growth caused by differences in lighting and wind speed in the different parts of the section were reduced to a minimum. Wind was continuous except during this changing around. After 4 to 5 days in the tunnel the plants were removed and their dry weight and leaf area were determined.

The initial sample, and the plants removed after exposure to wind, were treated in the same manner. The plants were removed from culture in the evening and the plants from each tin placed in separate glass containers kept moist with wet blotting-paper. The plants in these containers were stored at 2° C. until next day when the leaves were blueprinted. The plants were then dried in an oven at 95° C. for 24 hours, cooled in a desiccator, and weighed. Leaf areas were determined with a planimeter from the blueprints. Relative growth-rates (R.G.R.) and net assimilation rates (N.A.R.) were calculated according to the methods used by Blackman and Wilson (1951). R.G.R. is based on the dry weights of all the plants but N.A.R. and leaf area ratio (L.A.R.) are based on a sub-sample for which leaf area was measured. The number of plants in this sub-sample will be given in brackets after the total number of plants sampled. The L.A.R. calculated is that found at the end of the period of growth in the tunnel.

RESULTS

Rape

Rape has been grown in three similar experiments conducted in mid-June, late July, and mid-August. In all these experiments the plants entering the tunnel had been planted about a fortnight previously, their dry weight was approximately 30 mg., the length of the first leaf about 3 cm. and that of the second 1·3 cm. They were grown for 4 days in wind by which time they had increased approximately threefold in dry weight. A total of 175 (163) plants was sampled initially, 200 (152) from sections 2 and 8, and 300 (152) from sections 1 and 4. Plants were combined in fours for determination of dry weight and leaf area.

The growth analysis for rape is shown in Fig. 2 where each value is the average of the values found in the three experiments. The variation in L.A.R. is significant ($P < 0{\cdot}001$), that in N.A.R. and R.G.R. is not significant but that in R.G.R. would be expected by chance only once in ten groups of three experiments.

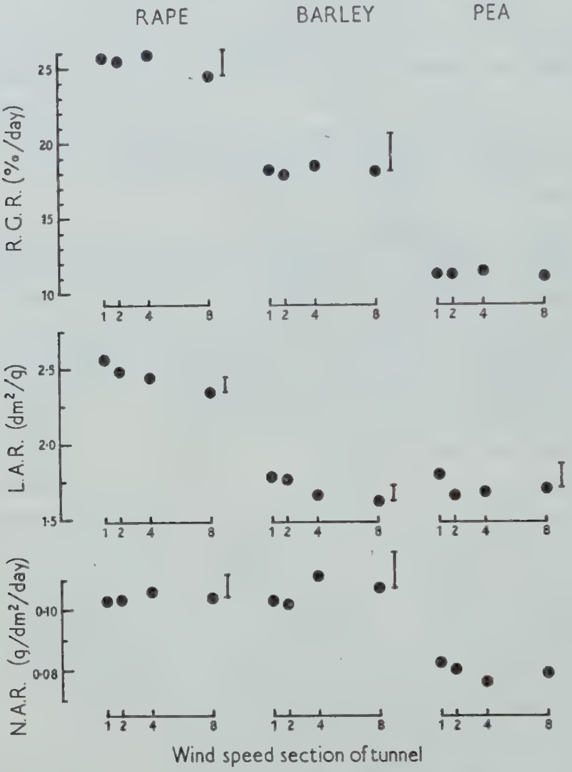

FIG. 2. Average values of relative growth-rate, net assimilation rate, and final leaf-area ratio for rape, barley, and pea grown in the four sections of the wind tunnel. The vertical line is the 5 per cent. significance level to be used in comparing the values obtained in sections 1 and 8 (the data for pea R.G.R. and N.A.R. were unsuited to the calculation of a significance level).

The plants in the highest wind speed were bent over by the wind and there was some leaf flutter. These effects were greatest at the front of the stand where the wind speed was greatest; they caused very little mechanical damage. There was no leaf movement at the lower wind speeds initially but by the time the plants were removed some of the leaves in the lowest wind speed showed slight flutter. The number of leaves formed was the same in all sections.

Barley

Barley has been grown in two similar experiments conducted in mid-June and at the end of July. The plants in each case entered the tunnel 10 days after sowing, when the first leaf was about 9 cm. long and had ceased growing, and the second leaf was about 4·5 cm. long. The plants were grown for 5 days in the wind in which time they increased about 2·5 times in dry weight. A total of 180 (120) plants was sampled initially, 160 (120) from sections 2 and 8, and 240 (120) from sections 1 and 4. Plants were combined in fours for determination of dry weight and leaf area.

The growth analysis for barley is shown in Fig. 2 where each value is the average of the values in the two experiments. The variation in L.A.R. is significant ($P < 0{\cdot}05$), that in N.A.R. and R.G.R. is not significant.

The plants in the highest wind speed were bent over, so reducing the height by from one-third initially to one-half finally (Plate). The growth form of the plants was similar in all sections but in the fastest wind speed (section 8) there was no leaf sheath visible between the first two nodes whereas in section 1 there was a distance of 1·5 cm. between the nodes. The plants in sections 2 and 4 were intermediate. Leaves tended to emerge earlier and to be more erect in the slower speeds.

Pea

Pea (variety Onward) has been grown in one experiment conducted in mid-August. The plants entered the tunnel 16 days after planting when they were 6 cm. tall and had opened their second leaf with leaflets. The plants were rather large and so were spaced twice as far apart as those of the other species. The plants remained in the tunnel for 5 days by which time they had nearly doubled in dry weight. Thirty-nine (20) plants were sampled initially and from sections 2 and 8, and 58 (20) from sections 1 and 4. Dry weight and leaf area were determined for each plant individually.

The growth analysis for this experiment is shown in Fig. 2. The variation found in N.A.R., R.G.R., and L.A.R. is not significant but that in L.A.R. would be expected by chance only once in seven experiments.

The pea plants showed very little effect of the wind they were experiencing. The plants in the fastest section tended to bend slightly with the wind. When these plants were exposed to wind from the opposite direction, after the daily turning around of the supporting lids, the stems tended to form an S-shaped bend of small angle.

Statistical Analysis of the Data

Rape and barley. The mean values found in each experiment were used in an analysis of the variance of the data for net assimilation rate and relative growth-rate. The mean values will on analysis give nearly the same ratios of mean squares and confidence limits as would be obtained if sample data were available since this was found to be so in analyses of the sample data for final dry weight, leaf-area, and leaf-area ratio. In these analyses the interaction between wind speeds and experiments was used to judge treatment effects. Variation in wind effect between experiments was random.

Pea. A single experiment was carried out and so the treatment effects were judged on the sample variance. Treatment effects on net assimilation rate and relative growth-rate were judged from the treatment effects on final dry weight, leaf-area, and leaf-area ratio. Analysis of the variance of the data for final dry weight and leaf-area shows that the differences found between different wind-speed treatments do not approach significance. Analysis of the variance of the data for final leaf-area ratio shows that the differences found between wind speeds would be expected by chance only once in seven experiments and so approach significance. These analyses indicate therefore that net assimilation rate and relative growth-rate do not show significant variation with wind speed.

In Fig. 2 a line to indicate the 5 per cent. confidence interval for judging the differences between the values found in sections 1 and 8 (Snedecor, 1956, p. 251) is shown for the data which have been subjected to an analysis of variance.

The results indicate no significant variation in R.G.R. or N.A.R. in any of the three species. Final L.A.R. fell significantly in rape and barley but not in pea. This fall is hardly apparent in the difference between the curves for R.G.R. and N.A.R. because these, in contrast to that for L.A.R., are averages over the whole experimental period. The results suggest that rape may be more affected and pea less affected by wind under these conditions.

DISCUSSION

The small effect of wind found in these experiments was not that expected from previous published work. The wind speeds found among the plants in sections 1 and 2 are below the 0·9 m./sec maximum used in the experiments of Deneke (1931), but N.A.R. in these sections does not show the increase in assimilation which Deneke found with increase in wind speed. The rape plants grown corresponded in height to the plants in the second to third categories of Wadsworth (1959). From his results with rape grown in sand culture in the wind tunnel used in these experiments, a decrease in growth over the whole range of wind speeds used would be expected. A decrease, at the higher wind speeds used, is also indicated by the results obtained by previous workers who have grown various plants for relatively long periods in artificial wind. How may these differences in the effect of medium wind speeds on growth be explained?

I suggest that the reduction in growth found in whole plants in wind is caused by shortage of water and that in my experiments this effect has been reduced by a plentiful supply of water to the roots.

Increase in transpiration per unit area of leaf surface (or per plant size) has been found as wind speed increases in all the long-term experiments where it has been satisfactorily measured (Martin and Clements, 1935; Wrenger, 1935–6; Rao, 1938). Such increases in transpiration will increase the diffusion pressure deficit in the leaves. Whitehead (1957) has reported leaf water-deficits in wind. He found deficits of 8 and 12 per cent. in three species of *Senecio* after 11 hours in a wind speed of 4 m./sec, and greater deficits at higher wind speeds. The plants were initially turgid, were grown in continually moist sand and artificially illuminated. Deficits of this size will reduce photosynthesis both directly (Rabinowitch, 1945) and by probable closure of the stomata (Stålfelt, 1955). Deficits will be greater when less water is available to the roots and when plants are insolated.

Thus under normal conditions, water-deficits sufficient to reduce growth will be readily produced by wind. The experiments of Deneke (1931) were conducted in nearly water-saturated air. The increase in assimilation he found up to the maximum wind speed he used of 0·9 m./sec is then to be expected as wind caused reduction in the length of the diffusion path for CO_2 to the leaf but transpiration was not sufficiently increased to cause effective increase in the leaf water-deficit. I have found a similar increase in growth-rate with wind speed, at the wind speeds used in the water culture experiments, in a single experiment in humid air (90 to 98 per cent. R.H.). Rape plants of the size grown in water culture were grown in sand culture in the two fastest wind speed sections of the tunnel (4 and 8). R.G.R., N.A.R., and L.A.R. were greater (R.G.R. and L.A.R. significantly so ($P < 0{\cdot}05$)) for plants grown in the higher wind speed. In air of more normal humidity growth-rate may be increased at low wind speeds (Heinicke and Hoffman, 1933; Wadsworth, 1959). At these low wind speeds the increase in transpiration caused by wind is not very great and the deficits produced will also be relatively small.

Leaf water-deficits may be reduced to a lesser extent by lesser increases in humidity or by increases in the availability of water to the roots. Either of these will therefore reduce the adverse effect of wind. The results of Wadsworth (1959) with sand culture show a much greater adverse effect of wind on rape than that which I have found in water culture where water is freely available to the roots. The results of Satoo (1948) indicate an increase in the adverse effect of wind when *Robinia pseudacacia* grown in pots was given less water. The results of Haas (1939) who records increase in the effect of wind on young avocado plants when small amounts of sodium chloride are added to the soil are of doubtful significance and are complicated by the great sensitivity of avocado to chloride.

These effects of wind on assimilation are summarized in Fig. 3. Wind shortens the diffusion path for CO_2 to the leaf and so increases the potential assimilation (curve A). Under humid conditions, where diffusion pressure deficits are small, assimilation (curve B) approaches this potential assimilation (Fig. 3 (i)). Decreased availability of soil moisture or increased evaporating power of the leaves (caused by lower relative humidity of the air or increased insolation) will, by increasing transpiration, increase the drying effect of wind and produce the effect shown in Fig. 3 (ii) and (iii). Here increase in diffusion pressure deficit has decreased assimilation sufficiently for higher wind speeds to reduce assimilation rate. Growth rate will be reduced more than assimilation rate because of the decrease in leaf area produced by increase in the water-deficit of the plant.

Variation in the effect of wind with climatic conditions and with availability of water to the roots of the plants is sufficient, I believe, to explain the differences in the effect of wind on growth found by different investigators.

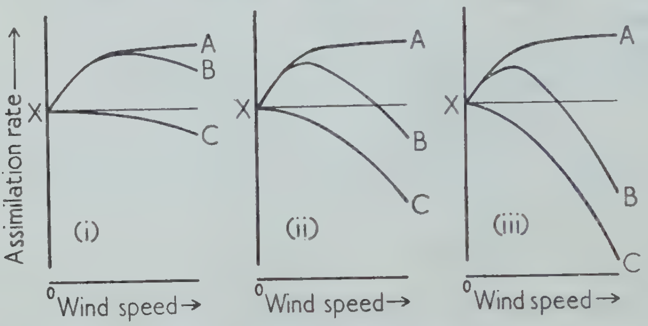

FIG. 3. Diagram to illustrate the predicted effect of moderate wind on assimilation. (i) Small drying effect of wind; high humidity of the air and sufficient soil moisture, (ii) medium drying effect, and (iii) large drying effect; low humidity and dry soil. Curve *A* suggests the potential assimilation in wind, curve *C* suggests the decrease in assimilation produced by the decrease in leaf water content caused by wind, and curve *B* represents the actual assimilation in wind; it is the resultant of curves *A* and *C*. X is the assimilation in calm air.

In my experiments with plants grown in water culture, wind adversely affected the water relations of the plants. This deficiency is indicated by the reduction in the size of plant parts (leaf area, plant height, and internode length) which was found as wind speed increased. Presumably the reduction in growth caused by this reduction in water content just balanced the increased assimilation to be expected from increase in the supply of CO_2 to the leaf surface.

The experiments discussed in this paper have been conducted at air speeds similar to those found in the field. It is hoped that they will provide a basis for the study of wind effect under more natural conditions.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the help I have received during the course of this investigation: from Dr. J. Warren Wilson who has supervised my work and given much helpful advice; from the University of Reading Research Board for a grant to cover the cost of the tunnel and some accessory apparatus; from the Department of Scientific and Industrial Research for a research studentship during the tenure of which this work was carried out.

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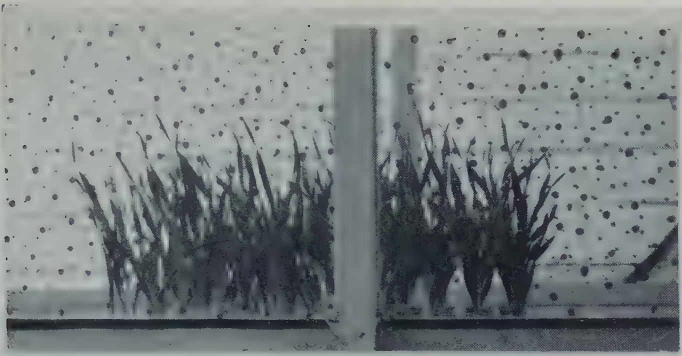
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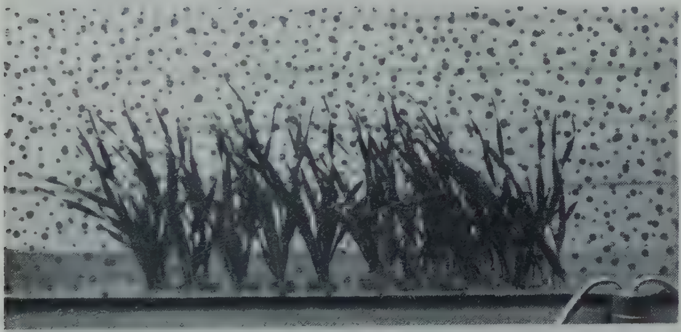
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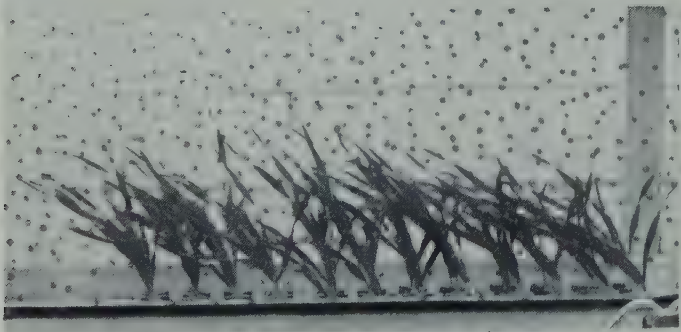
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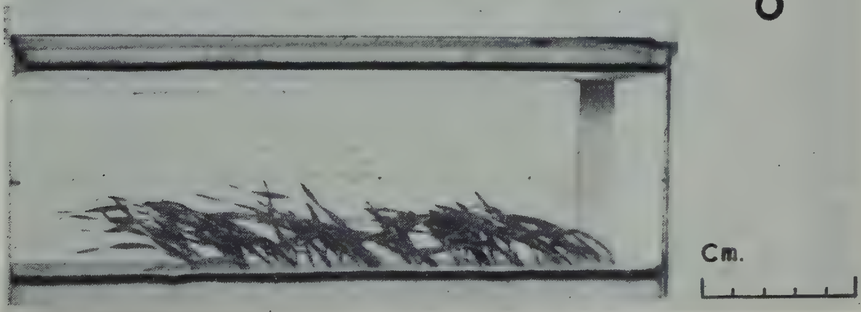
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EXPLANATION OF PLATE

Plants of barley growing in the four sections of the wind tunnel at the end of an experiment. The average growth analysis for this experiment, combined with that from one other similar experiment, is shown in Fig. 2. The spots of aluminium paint on the glass reduce the light in sections 1, 2, and 4 to that found in section 8 (80 per cent. full daylight). All the photographs are to the same scale.

Experiments on Banana Fruit Development

BY

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ABSTRACT

A number of synthetic auxins and auxin-antagonists were applied in lanolin emulsions to young banana fruits and their effects on fruit development and seed fertility estimated. Auxins stimulated the formation of edible pulp in both seedy and parthenocarpic bananas and induced varying degrees of seed sterility. Of the auxin antagonists tried, coumarin (contrary to a mistaken earlier report) stimulated parthenocarpy and induced seed sterility, three others were not or but slightly active, and thiourea markedly inhibited parthenocarpy. Study of the relation between size of mature fruit and seed content indicates that developing seeds stimulate the growth of pulp and that the amount of stimulation is roughly proportional to the level of parthenocarpy. Fruit development in the banana seems to fit in with Luckwill's general scheme of dual hormonal stimulus.

INTRODUCTION

THE ovary of an edible banana develops without external stimulus into a fruit that, at maturity, is packed with a starchy, parenchymatous pulp. The fruit is said to be vegetatively parthenocarpic and earlier investigations (Simmonds, 1953*a*) suggested that the behaviour is caused by the autonomous production of auxin in the young ovary, auxin which, in the seeded banana, is produced by the developing seeds. The evidence advanced in favour of this view was that: (1) α-naphthylacetic acid applied to the ovary of *Musa balbisiana* caused a definite (though sparse) development of pulp in the fruit; and (2) a known auxin antagonist appeared to reduce fruit size in an edible banana type. Both these observations have now been confirmed, though it will be shown below that the evidence earlier presented in favour of the second contention was faulty.

The work reported here was undertaken in order to extend the range of auxins and auxin antagonists used and to investigate the effects of the chemicals on seed fertility. In particular, it was hoped that one or more antagonists might be found which, by inhibiting pulp formation, might promote seed development in normally infertile edible types, thus greatly facilitating genetical and breeding experiments.

MATERIALS AND METHODS

Plant material. The following edible banana clones were used (classification after Simmonds and Shepherd, 1955):

AA Group:	'Pisang lilin' (I.R. 143, Type 32, 'Pisang lilan' of earlier publications).

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[Annals of Botany, N.S. Vol 24, No. 94, 1960.]

AAA Group: 'Red' (Type 7).
AAB Group: 'Mysore' (Type 6).
ABB Group: 'Pisang awak' (Type 12; 'Awak legor' of earlier publications).

And the following seeded types:

Musa acuminata subsp. *malaccensis* (Clone Selangor, I.R. 53).
Musa balbisiana (Clone Ceylon, I.R. 100).

Also the following clone of experimental origin:

J.349 (I.R. 275, a diploid AA type that originated from the cross 'Gros Michel' (AAA) × *Musa acuminata* (AA); slightly parthenocarpic but markedly seed fertile (Wilson, 1946)).

Methods. All applications were made in lanolin emulsion prepared by the method of Winklepeck and McClintock (1941). In the early phases of the work the emulsions were applied over the whole fruit surface but this interfered with normal fruit development as shown by the following results from 'Pisang lilin':

Treatment	Fruit volume at maturity (c.c.)
Control, untreated	20·8
Lanolin	16·0
Lanolin emulsion	14·3
Petroleum jelly	12·8

(Least significant difference (5%)—3·0 c.c.)

In all experiments described here (unless otherwise stated) applications were made as narrow streaks laid on with a fine paint brush, successive applications being made in the same place so that one-fifth or less of the fruit surface was covered with paste. Most of the experiments described here contained both a 'control' (untreated) treatment and a 'lanolin control' (lanolin streak only) and thus provide a means of assessing the effect of the lanolin streak as such upon fruit development. The average fruit volumes at maturity for these two treatments, in twelve experiments, were:

Control	Lanolin control
52·4	52·1

Eight deviations were in favour of the control and several of the differences were significant. The lanolin streak, therefore, has a somewhat variable but not large effect. When results are expressed as percentages in this paper they are percentages of the 'lanolin control' treatment.

All experiments were of randomized block type and plots were single fruits (fingers). One block (occasionally more, if the number of fruits per hand in relation to the number of treatments per block permitted) was laid out in each hand. Pollen was applied in paper-plasticine capsules. At maturity, bunches were dissected and fruit volumes measured by displacement in water. Seeds were extracted and counted as Good (G) or Bad (B), the former being well-formed seeds that sank in water, the latter variously malformed ones that floated in water. The distinction between Good and Bad seeds is not an absolute one, however. Statistical analyses of seed numbers were carried out after square-root transformation. Level of significance is indicated by the convention: *—5%; **—1%; ***—0·1%.

There follows a list of the hormones and hormone antagonists used, together with abbreviations adopted in this paper:

3IAA	3 indolylacetic acid
N24D	Sodium N-2, 4 dichlorophenoxyacetate
ANAA	α-naphthylacetic acid
BIBA	β-indolylbutyric acid
245T	2, 4, 5 trichlorophenoxyacetic acid
4CPA	4 chlorophenoxyacetic acid
A4CP	α-4 chlorophenoxypropionic acid
BNXA	β-naphthoxyacetic acid
DLTP	DL-tryptophane
CM	Coumarin
TU	Thiourea
IB	Tri-iodobenzoic acid
EC	Ethylene chlorhydrin
MH	Maleic hydrazide

RESULTS

a. Experiments on treated ovaries

The results of 27 experiments are reported here. They can be conveniently summarized under seven series, as follows:

Series	Plant material (expts.)	Treatments
1	'Mysore' (2), 'P. awak' (2), 'P. lilin' (2)	Nine auxins; 10,000 p.p.m.; applied at receptivity and weekly for 4 weeks.
2	'Mysore' (1), 'P. awak' (1), 'P. lilin' (1)	Five antagonists; 10,000 p.p.m.; applied at receptivity and weekly for 4 weeks.
3	'Mysore' (4), 'P. lilin' (2)	Coumarin; 1,000 p.p.m.; applied weekly over whole surface for life of bunch.
4	'Mysore' (1), 'P. lilin' (1)	Three antagonists; 1%; applied twice per week.
5	'Bluggoe' (1), 'Red' (1), 'P. awak' (1), J.349 (1)	Two antagonists; 1,000 p.p.m.; applied at receptivity and weekly for 4 weeks.
6	*M. acuminata* (2), *M. balbisiana* (1)	Two auxins, one antagonist; 1,000 p.p.m.; single treatment at receptivity.
7	*M. acuminata* (1), *M. balbisiana* (2)	N24D and TU; 1,000 p.p.m.; single applications 4 times—at receptivity and 1, 2, 4 days afterwards.

Series 1. Since all nine hormones and two controls could not be included in each block (the hands not being big enough to contain eleven treatments), ANAA was arbitrarily chosen as a treatment common to all experiments and it was applied with four out of the remaining eight hormones. Two experiments were done on each of three clones so that all hormones were tried on each clone and the results in Table 1 are the means of three experiments,

except for ANAA where they are the means of six. Seed set was negligibly small except in one experiment on 'Pisang awak'; the volume results for this experiment were corrected for seed content by use of the correction factor 0·85 c.c. per seed (see below).

Side effects were evident in most treated fingers: they included persistence of styles; curling of the fingers away from the site of application of the hormone (i.e. the hormone induced the expected local increase in length); scorching and cracking of the skin; local swelling and deformation. A rough general estimate of the intensity of these side effects is given in Table 1.

TABLE 1

Series 1—The Effects of Nine Synthetic Hormones on the Development of the Fruits of Three Edible Banana Clones

	Percent. of lanolin control		
Hormone	Ovary length at 10 days[1]	Volume at maturity[1]	Side effects[2]
N24D	166	201	++
4CPA	141	157	+
A4CP	146	145	++
BIBA	143	137	++
245T	157	132	+++
ANAA	127	116	+
BNXA	125	113	+
3IAA	104	108	+
DLTP	101	107	+

Notes: [1] Each entry the mean of three or six experiments (see text); 'Pisang awak' results corrected for seed contents (see Table 2).

[2] Cracking of skin, curling, and distortion: + slight; ++ moderate; +++ severe.

The results show that the most active hormone (N24D) doubled mature fruit volume; the biggest single increase recorded was for one experiment on 'Pisang awak' in which the N24D treatment almost exactly trebled the volume at maturity. The next most active hormone was 245T, as judged by its effect on ovary length at 10 days; it had a comparatively slight effect on mature fruit volume, however, almost certainly because of the severe damage it did. At the other end of the scale 3IAA and DLTP were but slightly or not at all active. Broadly, it is clear that the more active the hormone the more damage it does and that mature fruit volume is not, therefore, a safe indicator of activity. The most efficient hormone (at the chosen concentration) was 4CPA in the sense that it was active enough to promote growth without being so active as to cause severe damage. This point is well illustrated by two experiments on 'Mysore' (not otherwise reported here). N24D was applied in three concentrations, at receptivity and at one week, with these results (percent. of lanolin control):

Concentration	10 p.p.m.	100 p.p.m.	1,000 p.p.m.
Length at 10 days	119	140	166
Volume at maturity	115	152	120
Side effects	—	+	+++

The results of one of the 'Pisang awak' experiments (Table 2) illustrate the effect of five hormones on seed yield and the correction of volumes for seed content referred to above. (For the choice of 0·85 as correction factor see Sect. *b* and Table 9, below.) The results indicate clearly that the more active the hormone in promoting fruit growth, the more effectively it disrupts seed development.

TABLE 2

An Experiment (Series 1) on 'Pisang awak'. The Effects of Five Hormones on Fruit Volume and Seed Fertility.

Treatment	Volumes (c.c.)	Seeds (G+0·66B)	Volume correction (0·85 c.c. per seed)	Corrected volume	Volume percent.
Control .	43·0	6·0	5·1	37·9	—
Lan. cont. .	51·3	7·3	6·2	45·1	100
BNXA .	49·3	8·0	6·8	42·5	94
ANAA .	48·3	5·7	4·8	43·5	97
4CPA . .	76·0	1·3	1·1	74·9	166
A4CP . .	73·0	0·3	0·3	72·7	161
245T . .	77·3	0·0	0·0	77·3	172

Series 2. The effects of five antagonists on fruit growth of 'P. lilin', 'Mysore' and 'Pisang awak' are summarized in Table 3. Analysis of mature fruit volumes for the first two experiments showed that coumarin stimulated growth and that thiourea depressed it. The first experiment yielded no seeds, the second very few, the third, on 'P. awak', the numbers recorded in the last two columns of Table 3. The results suggest the existence of an inverse relation with fruit size, a volume-stimulating chemical depressing seediness and vice versa.

TABLE 3

Series 2—The Effect of Five Auxin Antagonists on Fruit Growth of Three Edible Bananas; each Entry for Fruit Size is the Mean of Three Experiments.

	Percent. of lanolin control		'Pisang awak' Seeds[2]	
Treatment	Length at 10 days	Volume at maturity[1]	Good	Bad
Lan. contr.	100	100	20·3	5·6
CM .	109	113	14·3	5·9
IB .	100	106	18·7	4·7
EC .	102	102	19·7	4·3
MH .	99	97	18·1	4·3
TU .	99	89	26·1	4·6

Notes: [1] Results for 'Pisang awak' corrected for seed content (cf. Table 2).
[2] 'Pisang awak' experiment only; analysis of $\sqrt{}$(total seeds) gave V.R. treatments (6, 36) = 2·31*; TU did not significantly exceed the lanolin control, nor the latter the coumarin treatment.

To conclude, coumarin stimulated growth and (probably) reduced seed yield—i.e. it acted as a hormone rather than as an antagonist under the chosen conditions of the experiments. Thiourea depressed growth and, the results suggest, slightly increased seed number. The other chemicals were not or but slightly active.

In the light of these results, an earlier finding (Simmonds, 1953*a*) that coumarin depressed growth of 'Pisang lilin' fruits must be discounted. The error arose from bad experimental design; control fruits were untreated rather than treated with lanolin emulsion and the depressing effect of the emulsion *per se* was, therefore, not revealed.

Series 3. Six experiments, two on 'P. lilin' and four on 'Mysore', were done. Table IV shows that coumarin stimulated fruit growth, mature fruits being some 10–12 per cent. larger than the controls. The only side effect noted was style persistence. Too few seeds were set to draw any certain conclusions as to the effect of coumarin in this respect but it is noteworthy that the few that were set (34 G+B in a total of 180 fruits) all occurred in control fingers. These results agree well with the results of Series 2, just described.

TABLE 4

Series 3—The Effect of Coumarin on Fruit Growth of Two Edible Bananas.

		Fruit volumes (c.c.)			Percent. styles persistent	
Clone	Experiments	Lanolin control	Coumarin	Coumarin volumes percent. of control	Lanolin control	Coumarin
'P. lilin'	2[1]	19·3	21·3	110	13	88
'Mysore'	4[1]	37·6	42·2	112	6	36

Note: [1] Joint analyses of volume results: for 'P. lilin', V.R. treatments (2,28) = 25·5***; for 'Mysore' V.R. treatments (2,172) = 138.0***.

Series 4. The two experiments of this series in effect repeat three of the treatments used in Series 2 but at roughly five times the dosage (24 as against 5 applications). The results agree very well for, once again, ethylene chlorhydrin was inactive, coumarin stimulated growth (but more than twice as much as at the lower dosage) and thiourea depressed growth very strikingly. A negligible number of seeds was set.

TABLE 5

Series 4—The Effect of Three Auxin Antagonists on Growth of Edible Banana Fruits.

	Mature volume[1] percent. of lanolin control	
Treatment	'Mysore'	'P. lilin'
CM	132	126
EC	106	99
TU	60	50

Note: [1] Analyses: V.R. treatments 'Mysore' (4,60) = 53·7***; 'P. lilin' (4,20) = 74·5***. In each CM > EC = lan. cont. > TU.

Series 5. The four experiments of this series were performed primarily to investigate the indication of Series 2 that thiourea might stimulate seed formation in edible bananas. The results (Table 6) confirm the depressing effect of thiourea on fruit volume and show that maleic hydrazide also has a depressing effect (that was not revealed by Series 2) but give no evidence that either substance stimulates seed formation—indeed, the reverse seems to be true.

TABLE 6

Series 5—The Effect of Two Auxin Antagonists on Fruit Volume and Seed Set in Four Edible Bananas.

	Control, per fruit		Treatments as percent. of control			
			MH		TU	
Clone	Volume[1] (c.c.)	Seeds (G+B)	Volume[2]	Seeds	Volume[2]	Seeds
J.349 (AA) .	15	13·2+10·5	71	61	81	80
'Red' (AAA) .	68	1·0+ 5·5	85	32	85	54
'P. awak' (ABB)	41	0·4+27·2	61	29	73	65
'Bluggoe' (ABB)	151	1·6+ 0·8	99	71	76	0

Notes: [1] Uncorrected volumes.

[2] Volumes corrected to zero seed content. Factors used (see below): for J.349, 0·58; for 'Pisang awak', 0·85; for 'Red' and 'Bluggoe' 1·35.

Series 6. The three experiments of this series were performed to test the effects on seeded bananas of two auxins and one antagonist which had all been found to be active on edible bananas (Table 7). Unpollinated and otherwise untreated ovaries of seeded bananas swell slightly and persist as empty shells; they contain neither seeds nor pulp. Both N24D and 245T induced parthenocarpy in both species tested, the fruits at maturity being two or three

TABLE 7

Series 6—The Effects of Two Auxins and One Antagonist on the Development of Fruits of Seeded Bananas. Three Experiments, Two on Musa acuminata, *One on* M. balbisiana.

		M. acuminata			*M. balbisiana*			Volumes percent. of control (means)
Treatment		Volume (c.c.)	Seeds (G+B)	Partheno-carpy[1]	Volume (c.c.)	Seeds (G+B)	Partheno-carpy[1]	
Unpoll.	Control	3·7	—	(+)[2]	8·3	—	(+)[2]	100
	N24D	8·6	—	+++	29·3	—	+++	298
	245T	7·2	—	++	27·3	—	+++	268
	TU	3·2	—	(+)	10·7	—	(+)	110
Poll.	Control	14·4	44	—[3]	70·7	273	—[3]	100
	N24D	10·4	9	++[4]	61·3	141	+[4]	82
	245T	7·2	0	+++[5]	45·0	98	++[5]	60
	TU	14·0	54	—[3]	68·0	267	—[3]	97

Notes: [1] Conventions thus: — nil or undetectable by reason of seediness; (+) trace; + slight; ++ moderate; +++ strong.

[2] Traces in a few fingers only, presumably a neighbourhood effect.

[3] All fingers seedy, not at all parthenocarpic.

[4] N24 Dtreatments: 3/11 fingers in *acuminata* expts. seedy (mean content 34), remainder parthenocarpic and seedless; 2/3 seedy in *balbisiana* (mean content 212).

[5] 245T treatments: all 11 fingers in *acuminata* expts. seedless and parthenocarpic; 1/3 seedy in *balbisiana* (content 273), the other two parthenocarpic and nearly seedless (5 and 17 seeds).

times as big as the controls but only about half as big as fruits that had been pollinated and were, therefore, seedy; the level of induced parthenocarpy was therefore quite low, as in earlier experiments (Simmonds, 1953*a*, Fig. 6). On pollinated fruits the auxins had the same effect that they had on seedy edible bananas (Table 2): they effectively disrupted seed development, though the effect was somewhat irregular, some fingers being nearly normal as to seed content, others quite seedless and parthenocarpic.

Thiourea had no evident effect on the size of unpollinated ovaries. Nor did it affect seediness in *M. balbisiana*. In *M. acuminata*, TU-treated fruits did appear to be somewhat more seedy than the controls (V.R. [2,27] = 4·19*) but the effect was significant in only one out of the two experiments (t [27] = 3·20** and 0·06).

Series 7. As in the previous series, N24D disrupted seed development and induced parthenocarpy. In *M. balbisiana* thiourea affected seediness in the sense that the two experiments were significantly heterogeneous (V.R. [1,50] = 5·73*) though in neither did TU-treatments differ significantly from the controls. In *M. acuminata* an apparent depressing effect of thiourea on seediness was also non-significant (t [15] = 0·50).

TABLE 8

Series 7—One Auxin and One Antagonist on Seeded Bananas, Pollinated; Three Experiments, One on Musa acuminata, *Two on* Musa *balbisiana.*

	Percent. of control			
	acuminata		*balbisiana*	
Treatment[1]	Volume	Seeds	Volume	Seeds
N24D	61	1	85	15
TU	80	80	95	100

Note: [1] There were four times of application of each substance (0, 1, 2, 4 days) but no trends were apparent and the figures here are means of the four times.

Taken jointly, series 6 and 7 provide evidence that thiourea slightly depressed fruit size independently of any possible effect on seed content. In the six experiments concerned, volumes of TU-treated fruits were corrected to the volumes they would have had if they had contained the same number of seeds as the appropriate controls (cf. Table 2). The correction factor used was 0·235 c.c. per seed (see below). The calculated differences (controls *minus* corrected TU-treatments) were:

M. acuminata: +4·0, +1·1, +0·6
M. balbisiana: +0·8, +6·8, −0·1

suggesting that the treated fruits were on the average about 2 c.c. smaller than the controls.

b. The relation between size and seed content

It has been shown that the size of mature seeded bananas is proportional to the seed content and that the regression coefficients are 0·23–0·24 c.c. per seed; since the coefficients are much (3–5 times) larger than the volume of a seed, it follows that seeds have a stimulative effect on development. A similar effect has been found in edible bananas where, however, the regression coefficient was found to be 1·35 c.c. per seed—that is, a seed has six times as great a stimulative effect in an edible maternal environment as it has in a seeded one. In the determination of this latter coefficient, rather heterogeneous material was used; the majority of the clones tested were highly parthenocarpic and nearly seed sterile but a few were otherwise. It therefore seems worth while to segregate some of the latter to see whether the same coefficient applies as to the highly parthenocarpic clones. 'Pisang awak' and J.349 were chosen and Table 9 shows the results. Also included in this table are the results of an experiment on a recent introduction to the I.C.T.A. collections, I.R. 433, *Musa acuminata* subsp. *banksii* from Samoa. Its inclusion followed an observation by K. Shepherd that unpollinated fingers were thinly parthenocarpic, though the plant was in other respects an ordinary wild species which was highly seed fertile if pollinated.

TABLE 9

Determinations of Volume–Seed Regressions in Bananas (b, *c.c. per seed*)

Plant(s)	Fruit development	b[2]	stimulative effect[3]	Source
M. acuminata .	Seeded	0·23	4	Simmonds (1953*a*)
M. balbisiana . .	Seeded	0·24	4	,,
J.349 . . .	Seeded, v. sl. parth.	0·58	10	This paper
M. acuminata subsp. *banksii* . .	Seeded, thinly parthenocarpic	0·71	12	,,
'Pisang awak' . .	Moderately parthenocarpic, somewhat seed fertile	0·85	14	,,
Misc. edible clones[1]	Fully parthenocarpic, ± sterile	1·35	23	Simmonds (1953*a*)

Notes: [1] Heterogeneous, as indicated in text, but the great majority highly parthenocarpic.

[2] Regression coefficients, volume increments (c.c.) per seed, effective seed number being estimated as $(G+0{\cdot}66\,B)$.

[3] Factor by which regression coefficient exceeds average seed volume (different for *M. acuminata* and *M. balbisiana* but taken here to be average 0·06 c.c.).

The results indicate that the stimulative effect of a seed on fruit development is proportional to the level of parthenocarpy, being least in seeded bananas and greatest in highly parthenocarpic clones.

DISCUSSION

In recent years several instances have been found in which growth of a plant tissue is initiated and maintained by a dual chemical stimulus, one component stimulating cell division, the other cell enlargement. Systems of this kind have been discovered in three (superficially) very diverse tissues: potato tuber tissue (Steward and Caplin, 1951); crown gall tissue (Braun and Naf, 1954; Braun, 1956*a*, *b*); and in apple fruit tissue (Letham, 1958) (see also a review by Steward and Shantz, 1959).

Applying this concept of a dual chemical control of tissue growth to a discussion of fruit growth, Luckwill (1957) suggested that there were two principal stimuli to development. An essential initial impulse is, he suggests, provided by division-stimulating substances of the coconut milk factor (CMF) type which are produced by the young endosperm. Subsequent growth, Luckwill suggests, is carried on under the stimulus of auxin-type hormones which are primarily concerned with cell enlargement. This scheme offers a plausible explanation of the development of strawberries and pome fruits in which applied auxin stimulates growth only if fertilization and at least a limited degree of seed development has occurred. Fruits in which a high level of parthenocarpy can be induced by auxin treatment alone (e.g. the tomato) may be those in which growth of the post-flowering ovary occurs primarily by cell enlargement rather than by cell division.

In the bananas, both CMF and an auxin-type hormone (probably IAA itself) have been detected (Steward and Simmonds, 1954; Nichols, 1958) though no detailed quantitative sampling has yet been done. Auxins applied externally in lanolin pastes induce a moderate degree of parthenocarpy in unpollinated seeded bananas and stimulate pulp growth in parthenocarpic types (Simmonds, 1953*a* and the present work). Complementary to this is the observation that the auxin antagonist, thiourea, inhibits pulp growth in edible (probably also in seeded) bananas. Jointly, these findings seem to offer support for Luckwill's contention that, in the bananas as in other plants, two systems of stimuli are responsible for initiating and maintaining fruit development. In their reaction to applied auxins, the seeded bananas appear to be intermediate between the pome fruits and the tomato, implying, perhaps, that they are only partly dependent on seeds as an early source of division-stimulating hormones. This raises the possibility that a higher degree of parthenocarpy could be induced by means of CMF-auxin combinations than can be induced by the use of auxins alone (not only in the bananas, but also in other plants which have proved resistant to simple auxin applications).

In physiological-genetic terms the parthenocarpic would seem to differ from the seeded banana in possession of two synthetic systems which are autonomous in the sense of being independent of the presence of developing seeds; the finding that at least four complementary dominant genes are necessary for the expression of parthenocarpy in *acuminata* diploids (Simmonds, 1953*b*) is in good agreement with this view.

The observation that developing seeds have a stimulative effect that is roughly proportional to the degree of parthenocarpy expressed in the maternal environment can not readily be interpreted at the moment; the effect is, however, so large and regular that some explanation of it must be offered by any satisfactory hypothesis of the mechanism of banana fruit development.

Applied auxins not only stimulate the development of starchy parenchyma in the fruit, they also disrupt seed development, an effect which has been put to horticultural use in the case of the apple, where ANAA sprays are used to cause seed abortion, hence cessation of fruit growth, and hence thinning of an over-abundant crop. In the parthenocarpic banana, seed sterility certainly has many and complex causes, both genetical and physiological in nature. The hypothesis that autonomously produced auxin was at least a component of the physiological causes of sterility led to the experiments described here on auxin antagonists. Though the results in relation to fruit growth are unequivocal—thiourea certainly inhibits parthenocarpy—the results in relation to seed development are not: the most that can be said is that there is an indication that thiourea promotes seed fertility sporadically, in some clones and on some occasions. That the effect should be variable with clone and occasion is intelligible on cytological grounds (Simmonds, 1960). The fertility of a parthenocarpic banana may or may not be limited by the frequency of occurrence of functional embryo sacs; if embryo sacs are limiting, it is not to be expected that chemical treatments applied at receptivity could promote seed development but if they are relatively numerous (and if fertilization is adequate), then external treatments might well be effective. The point is of great practical importance to the banana breeder because a treatment which permitted the development of only one more embryo sac per bunch (say 1/30,000 ovules) of 'Gros Michel' would nearly double the gross seed yield. The experiments with thiourea, inconclusive as they are, therefore suggest a potentially very valuable line of investigation.

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The Respiration Rate of Tomato Stem Tissue Infected by Tomato Aucuba Mosaic Virus

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ABSTRACT

The oxygen uptake of stem tissue slices of *Lycopersicum esculentum* var. 'Stonor's Moneymaker', in 0·035 M. Sorenson phosphate buffer was measured manometrically; the optimum pH was found to be 5·2. With tomato-stem tissue systemically infected by virus (tomato aucuba mosaic), the respiration rate was always below that of healthy tissue when equal numbers of respiratory systems were compared. When the respiration rate was expressed in terms of oxygen uptake per unit fresh or dry weight, the respiration rate of the virus-infected tissue could be greater or less than that of the healthy tissue depending on the growth conditions of the experimental material. Under conditions favouring the complete development of disease symptoms, virus infection decreased the fresh and dry weight of the cell. For this reason fresh weight and dry weight were not considered suitable criteria to express the respiration rates as these units contained different numbers of respiratory systems in healthy and virus-infected tissue.

INTRODUCTION

A CONSIDERABLE amount of work has been carried out on the effect of virus infection upon the respiration rate of the host-plant. Most of this work has been concerned with changes in respiratory activity of tobacco or tomato leaves following infection with tobacco mosaic virus. It now appears to be established (Wynd, 1943), that immediately following infection the respiration rate rises. However, whether the respiration rate of systemically infected tissue is greater, less, or the same as comparable healthy tissue is a matter of dispute. The work discussed in the present paper forms part of an investigation of the relationship between host-plant respiration and virus multiplication (Merrett, 1959), and is concerned with the respiration rate of tomato stem-slices obtained from plants systemically infected with tomato aucuba mosaic virus (T.A.M.V.) grown under conditions favourable to the development of the disease symptoms.

MATERIAL AND METHOD

The tissue used in these experiments was slices of the stem of tomato *Lycopersicum esculentum*, var. 'Stonor's Moneymaker' grown under normal glass-house conditions during the months April to June. The stem was sectioned by means of a hand microtome, sections being cut at 300 μ in thickness.

The apparatus employed for measuring gaseous exchange was a Braun

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[**Annals of Botany, N.S. Vol. 24, No. 94, 1960.**]

Rotary Warburg apparatus. Respiration rates were determined by Warburg's direct method. Measurements were made at a temperature of 30±0·02° C. In most cases oxygen-uptake was expressed as μl. of gas taken up per hour per gramme final fresh weight. The procedure in a typical experiment was as follows: the slices of tissue were washed once with distilled water and then placed in a Warburg flask containing 3·0 ml. of phosphate buffer of the required composition. 0·2 ml. of 20 per cent. aqueous potassium hydroxide and a roll of Whatman No. 40 filter-paper were put in the centre well of the flask. The manometer and flask were placed in the apparatus and brought to equilibrium. Readings were taken at half-hourly intervals over the period of the experiment, which was normally 6 hours. At the end of this time the tissue was removed, dried on filter-paper and weighed to give the final fresh weight. A value for the dry weight was obtained by drying the tissue at 100° C. for 24 hours.

TABLE I

The Respiration Rate of Healthy and Virus-infected Tomato Stem Tissue

Plants grown during May and June. Stem sections 300μ in thickness, suspended in 0·035 M. Sorenson buffer pH 5·2. respiration measured over 6 hr. period

	Respiration rate, oxygen uptake/μl., gram fresh weight/hour							
	Healthy tissue				Virus-infected tissue*			
Experiment number	1	2	3	4	1	2	3	4
Apex . . .	7·5	32·7	84·0	50·5	11·0	151·3	150·0	142·5
Internode 3 . .	7·0	96·1	22·1	49·4	21·0	178·6	66·8	95·1
Internode 2 . .	7·3	20·0	37·2	40·4	8·0	88·5	55·0	75·0
Internode 1 . .	16·0	47·9	83·0	42·6	14·3	53·9	59·5	76·0

* Respiration rate of infected tissue greater than that of healthy tissue. Difference significant at level $P = 0{\cdot}001$.

In a preliminary series of experiments some of the conditions affecting the oxygen uptake of tomato stem slices were investigated. Although Caldwell and Meiklejohn (1937) recommended longitudinal sectioning of the tissue it was found that transverse sectioning of the tissue was more convenient and did not materially affect the respiration rate. The optimum oxygen uptake occurred at pH 5·2 which is about the same pH as that of extracted tomato stem sap. The concentration of phosphate buffer of the suspending medium had a considerable effect on oxygen uptake, maximum oxygen uptake occurring in 0·035 M. Sorenson phosphate buffer. Most experiments were carried out at that pH and with that buffer as giving optimum oxygen uptake.

EXPERIMENTAL

The respiration rates of healthy and T.A.M.V.-infected tomato stem tissue were compared. Young tomato plants of variety 'Stonor's Moneymaker' were inoculated at the two-leaf stage with expressed sap from tobacco plants, *Nicotiana tabacum* var. White Burley systemically infected with T.A.M.V.

The respiration rate was measured when the plants had reached the 6–7-leaf stage and compared with that of healthy controls grown under the same conditions. The results of this experiment are given in Table 1.

These plants were grown during the summer and the virus-infected plants showed maximum development of disease symptoms. From the results given in Table 1, it is seen that when oxygen uptake is measured in phosphate buffer and is expressed as oxygen uptake per gramme fresh weight, the virus-infected tissue shows a far greater respiration rate. The results show a considerable day-to-day variation in the actual respiration rates of the tomato stem slices. This was also observed by Link, Klein, and Barron (1952). Thus, even though the respiration rate of the virus-infected tissue was greater than that of the healthy in any one experiment, this could be reversed if, for instance, the respiration rate of the virus-infected tissue in Expt. 1 were compared with the respiration rate of the healthy tissue in Expt. 2 measured 2 days later. This variation necessitates measuring the respiration rate of healthy and virus-infected tissue at the same time.

TABLE 2

The Effect of Virus Infection on the Size of Tomato Plants

Plants inoculated at the two-leaf stage, grown during May and June

Overall height	Healthy control 20 cm.		Virus-infected plant 14 cm.	
Internode	Length (cm.)	Diameter (cm.)	Length (cm.)	Diameter (cm.)
Apex 5	2·5	0·5	2·0	0·2
4	3·0	0·5	2·0	0·3
3	3·0	0·8	2·0	0·4
2	3·0	0·8	2·0	0·4
1	3·5	0·8	2·0	0·5

The oxygen uptake of virus-infected and healthy tissue in relation to the number of cells present

In many of the recorded respiration studies the results are expressed on a fresh-weight or dry-weight basis. The probable reason for this is that these quantities are relatively easy to measure and, what is more important in most cases, unit fresh weight or dry weight contains the same number of respiratory units for a given tissue.

However, it appears that infection of the tissue with some viruses may affect these considerations. The effect of T.A.M.V. on the tomato plant is considerable. When temperature and light conditions are favourable to the development of the virus, the plant shows stunting and a spindling habit besides the development of mosaic markings. This can be shown by comparing a diseased plant inoculated at the two-leaf stage with a healthy control grown under the same conditions (see data in Table 2). This difference in size is not the result of different development. It was shown by Caldwell

(1934) that the development was not affected, as healthy and T.A.M.V.-infected plants of the same age grown under the same conditions had the same number of leaves and flower trusses. This can also be seen by sectioning healthy and diseased plants and counting and measuring the cells. If this is done, it is found that internodes of the same age of healthy and virus-infected tissue contain equal numbers of cells but that the size of the cells differs (Merrett, 1959). The reduction in size of virus-infected plants is the result of a reduction in cell size rather than cell number.

TABLE 3

The Effect of Virus Infection on the Fresh Weight and Dry Weight of Tomato Stem Internodes

Plants inoculated at two-leaf stage, grown during May and June. Dry weight obtained by drying at 100° C. for 24 hr.

Mean of 12 plants	Fresh weight (g.)		Dry weight (g.)		Wt. of diseased internode expressed as percentage weight of healthy internode	
Internode	Healthy	Diseased	Healthy	Diseased	Fresh weight	Dry weight
Apex 7	0·76	0·46	0·55	0·03	60·5	5·6
6	1·57	0·71	0·11	0·04	45·2	36·3
5	2·74	1·28	0·19	0·06	46·7	31·5
4	3·21	1·57	0·26	0·07	48·9	26·9
3	2·71	1·11	0·25	0·06	40·9	24·0
2	2·64	1·66	0·26	0·10	62·8	38·4
1	1·62	1·29	0·17	0·09	79·6	52·9

The effect of time of inoculation on internode development

From this consideration arises the possibility that reduction in cell size may be accompanied by a reduction in weight. To investigate this possibility tomato plants were inoculated with T.A.M.V. at the two-leaf stage and allowed to grow until they reached the six-leaf stage when the internodes were weighed to obtain the fresh weight and then dried at 100° C. for 24 hrs. to give the dry weight. These results are given in Table 3. The fresh weight of a virus-infected internode was from 40 to 79 per cent. of the fresh weight of a healthy internode. Since there is a reduction in the overall fresh weight and dry weight of each internode in infected tissue, there is a corresponding reduction in the fresh weight of the cells of infected tissue when compared with those of the healthy control—in other words, unit fresh weight or dry weight of virus-infected tissue will contain a greater number of cells than comparable healthy tissue if development of the tissues has taken place under conditions favouring the development of disease symptoms.

These results were confirmed in an experiment in which the effect upon fresh and dry weight of the stage at which infection took place was investigated. Tomato plants grown during April, May, and June 1957, were inoculated with T.A.M.V. at the cotyledon, two-leaf, and four-leaf stages and compared with the controls when all the plants had reached the nine-leaf stage. It was found that the effect of virus infection upon tomato stem internodes was considerable. The difference between the fresh weight of healthy and of virus-infected internodes was highly significant ($P > 0·001$). Similar results were obtained from dry weight, the difference between dry weight of healthy and virus-infected internodes being significant ($P > 0·001$). The stage of development of the plant when infection took place was also significant. There was a greater reduction in fresh weight and dry weight of internodes formed after infection than those developed before. The differences in internodes present before infection compared with those formed after infection were significant on a fresh weight ($P > 0·01$) and on a dry weight basis ($P > 0·05$).

TABLE 4

The Effect of Stage of Virus Infection on the Fresh Weight and Dry Weight of Tomato Stem Internodes

(Plants grown during April, May, and June)

Mean of 12 plants	Weight of diseased internode expressed as percentage weight of healthy internode					
	Plants inoculated at the cotyledon stage		Plants inoculated at the 2-leaf stage		Plants inoculated at the 4-leaf stage	
Internode	Fresh Wt.	Dry Wt.	Fresh Wt.	Dry Wt.	Fresh Wt.	Dry Wt.
Apex 8	86·2	8·6	7·0	23·2	56·9	48·9
7	77·6	11·9	20·7	12·2	57·6	55·2
6	67·5	8·9	17·9	53·4	75·7	60·9
5	75·6	11·6	26·7	60·5	89·9	71·2
4	77·5	8·0	26·9	60·8	78·2	65·4
3	64·3	6·9	19·6	47·4	97·5	90·8
2	56·4	9·6	27·1	40·3	78·8	87·8
1	67·7	8·6	44·9	49·8	81·6	77·6

A reduction in fresh and dry weight in internodes present before infection did, however, occur when these internodes were compared with those of healthy controls. This reduction was not nearly so great as that in internodes formed after infection. Another feature of these results is the effect of the stage of infection on the dry weight of the internode. It can be seen that with infection at the cotyledon stage the dry weight of the infected internode is from 6·9 to 11·9 per cent. of the dry weight of a healthy internode, at the two-leaf stage 12·2 to 60·8, and at the four-leaf stage 48·9 to 90·8 per cent. of the dry weight of a healthy internode. It was suggested by Caldwell (1934) that the virus had no effect on preformed chloroplasts, although it was thought by some workers that this may not be the case, as yellowing of inoculated leaves occurs. However, if preformed chloroplasts are not affected by the virus, the number of active chloroplasts and photosynthetic ability of the plant depends on the age at which it was inoculated. This could well account for the greater reduction of dry weight of infected internodes with earlier age of infection. But this may not be the complete explanation, as the fresh weight shows variation as well as the dry weight. Thus, these results may be the result of variation in light intensity and temperature during the growing season of the plants. What does remain certain is that virus infection is accompanied by considerable changes in fresh weight and dry weight of the cells when conditions favour the development of disease symptoms.

With these facts in mind, respiration experiments were designed to eliminate differences in respiration resulting from different numbers of cells in unit fresh weight of healthy and virus-infected tissue.

TABLE 5

The Respiration Rate of Healthy and T.A.M.V.-Infected Tomato Stem Tissue Expressed on a Fresh-weight and Internode basis

Plants inoculated at cotyledon stage and grown during June and July. Stem sections, 300µ thickness, suspended in 0·035 M. Sorenson buffer pH 5·2

Internode	Respiration rate µl. O_2/g. fresh weight/hour measured over 6 hr. period		Respiration rate µl. O_2/internode/hour measured over 6 hr. period	
	Healthy tissue	Virus-infected	Healthy tissue	Virus-infected
Apex	84·0	150·0	11·0	6·0
3	22·1	66·8	8·0	7·0
2	53·6	56·0	70·0	13·0
1	83·3	59·0	75·0	23·0

Comparison of respiration rates per internode

As it has been shown that internodes of healthy and virus-infected plants did not differ in cell number, comparable internodes of healthy and diseased tissues were sectioned and the oxygen uptake of the sections from each internode recorded. As before, the plants used showed complete development of disease symptoms. They had been inoculated at the cotyledon stage and used when they had reached the eight-leaf stage. It can be seen that on a fresh-weight basis the virus-infected tissue respired at a greater rate than the healthy, as had been shown previously. On an internode basis the respiration rate of virus-infected tissue was below that of healthy tissue. Thus it is apparent that in virus-diseased tissue the respiration rate per cell is below the respiration rate of a healthy cell. When the results are expressed on a fresh-weight basis this is reversed, because unit fresh weight of virus-infected tissue contains a greater number of cells than unit fresh weight of healthy tissue and consequently a greater number of cells than unit fresh weight of healthy tissue and consequently a greater number of respiratory systems making their contribution to the overall respiration.

These results were confirmed in another series of experiments when tomato plants were inoculated at the two-leaf stage and used at the eight-leaf stage. Unfortunately these plants were grown in the autumn and measurements carried out in September, and although the diseased plants showed mosaic symptoms they did not show the extensive dwarfing of plants grown under the optimum conditions for disease expression. The results are expressed as oxygen uptake per unit fresh weight, per unit dry weight, and per internode per hour and are given in Table 6. The difference in respiration rates on an internode basis is significant. However, in this experiment the differences in respiration rate between healthy and virus-infected tissues expressed on a fresh-weight and dry-weight basis are not significant.

TABLE 6

The Respiration Rate of Healthy and Virus-infected Tomato Stem Tissue Expressed on an Internode, Fresh-weight, and Dry-weight Basis

Plants inoculated at the two-leaf stage and grown during August and September. Stem sections 300 μ in thickness, suspended in 0·035 M. Sorenson buffer pH 5·2

	μl. O_2/hr./g. fresh weight		μl. O_2/hr./mg. dry weight		μl. O_2/hr./internode	
	Total oxygen uptake for 15 samples	Mean oxygen uptake per sample	Total oxygen uptake for 15 samples	Mean oxygen uptake per sample	Total oxygen uptake for 15 samples	Mean oxygen uptake per sample
Healthy tissue	330·0	22·0	41·64	2·76	427·5	28·5
Virus-infected tissue	254·5	17·3	39·52	2·63	108·0	7·2*

* Respiration rate of virus-infected tissue less than that of healthy when expressed on an internode basis; $P = 0{\cdot}05$.

DISCUSSION

The effect of infection with T.A.M.V. upon the respiration of the tomato plant can, for the sake of simplicity, be considered as two separate effects. These are (1) the effect of infection on the units in which the respiration rate is usually expressed, that is dry and fresh weight, and (2) any actual change in the respiration rate of the plant which is not indirectly the result of (1).

The present experiments were carried out on infected plants which were completely diseased. From the data presented it is shown that both the fresh weight and dry weight of an equal number of cells was considerably less in virus-infected tissue. Since the dry weight per cell is lower, the fall is not merely a result of a lower water content of the diseased tissue. For instance, the results given for tomato plants inoculated at the cotyledon stage show that the dry weight of the internodes was only 10 per cent. that of the healthy internodes. Similar results obtained by Caldwell (1934) showed that for healthy and virus-infected tomato plants grown under comparable conditions, the healthy plant had a fresh weight of 88·0 g., the infected 20·1 g. The percentage dry matter of the healthy plant was 12·9 and that of the infected plant 7·8 per cent. Thus, following virus-infection under conditions favouring disease-development the dry weight is decreased as well as the fresh weight. In fact, in the present experiments with healthy and diseased tissue, it was found that the percentage dry weight of healthy tissue was between 7 and 14, while for virus-infected tissue it was between 6 and 9. Owen (1955*b*) states that 'for *a priori* reasons, dry matter is the most logical basis (to express respiration results), as the water in the leaves does not contribute to respiration'. However, there is no *a priori* reason why most of the dry weight should contribute to respiration, since most of the dry matter left after drying consists of the structural elements of the tissue, such as the cell walls. Also, it has been shown in the experiments recorded here that there is a decrease in dry weight of the cell following virus infection. This decrease is considerable so that when referring respiration rates of healthy and virus-diseased tissue to dry weight basis, a true comparison between equal numbers of respiratory systems is not obtained. Similar reasoning shows that basing the comparison on fresh weight is no better. Thus, for a true comparison to be made, it is essential to express the respiration rate in terms of the plant, leaf, or internode, or some other unit which contains equal numbers of respiring cells. This approach was used by Porter and Weinstein (1957) in their study of biochemical changes induced by thiouracil in cucumber mosaic virus-infected and non-infected tobacco plants.

Thus, with virus-diseased plants two extreme conditions are possible. With plants grown under conditions favouring disease-development, unit fresh weight or dry weight of diseased tissue will contain many more cells than comparable healthy tissue; on the other hand although no data have been obtained for infection under conditions not favouring disease-development, it is probable that with plants not showing disease symptoms, unit fresh weight or dry weight of virus-infected tissue will contain approximately as many cells as healthy tissue. From this data it is not difficult to envisage virus-infected tissue having a greater, lesser, or the same respiration rate as healthy tissue when the results are expressed on a fresh-weight basis. From the results presented it can be seen that on a fresh-weight basis virus-infected tissue respires at a greater rate than healthy tissue. However, when results are expressed on a cell for cell basis, the respiration rate of the virus-infected tissue is always below that of healthy tissue when the plants have been grown under conditions favouring disease-development. Although the effect of environmental conditions on the fresh and dry weights of the plants has been investigated, no attempt has been made to investigate the effect of such environmental factors as light intensity on the respiration rate. It is quite possible that besides affecting the development of disease symptoms, changes in light intensity might differentially affect the respiration rate of healthy and virus-infected tissue. Owen (1955*a*) found that the effect of tobacco mosaic virus infection on the respiration of detached tobacco leaves in the period immediately after inoculation differed in plants grown at different times of the year. In winter infection increased respiration rates, in summer it decreased them. Thus many factors must be taken into consideration when investigating the effect of virus infection on plant respiration.

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Short-day and Low-temperature Induction in *Lolium*

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ABSTRACT

The requirements for floral induction of several outbreeding populations of *Lolium perenne*, *L. multiflorum*, and *L. rigidum* have been studied. Floral induction can be brought about by either low temperature (0–3° C.) or short day (8 hr.) independently, but short day is ineffective at low temperatures.

The exact inductive requirement varies with the population. The summer-annual Westerwolds ryegrass needs neither cold nor short day, the winter-annual Wimmera ryegrass shows a quantitative response, while the perennial varieties from north-west Europe have an obligatory requirement for either cold or short day before floral induction. This relation between the annual or perennial habit and the inductive requirement was confirmed in a number of Mediterranean collections of *L. perenne* and *L. rigidum*.

Considerable genetic variation in inductive requirement could be detected within each outbreeding population, and rapid response to selection proved possible in Wimmera, Irish, and Kent ryegrass.

INTRODUCTION

IT has been known for some time that in *Lolium* the perennial species usually require exposure to winter conditions before floral induction can occur, while this is not so for the annuals which flower readily after a spring sowing (Cooper, 1951, 1957; Wit, Margadant, and Dilz, 1955; Wycherley, 1952). This winter requirement can be satisfied artificially by low-temperature exposure (0–3° C.) in the refrigerator, and it has been possible to use this treatment for the rapid identification and classification of varieties (Cooper 1957). Pilot work, however, indicated that the effects of low temperature alone were not sufficient to account for complete winter induction in the field, particularly in the extreme perennial varieties, and that short days might also be effective, either alone or combined with low temperature.

The present paper, therefore, is concerned with the response to short-day induction of populations of *Lolium* from different climatic and agronomic origins. It also gives information on the genetic control of this character and its response to selection.

SHORT-DAY AND LOW-TEMPERATURE INDUCTION

Two experiments, one autumn-sown 1956 and the other spring-sown 1958, were carried out to assess the relative importance of short-day induction, either alone or in combination with low temperature.

Autumn-sown plants 1956. The following outbreeding populations of *Lolium* were chosen to cover the agronomic and climatic range in the genus;

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

all are diploid ($2n = 14$) and interfertile. They are listed below in order of perenniality.

(1) *L. multiflorum* Lam. Westerwolds ryegrass: summer annual, from the Netherlands.
(2) *L. rigidum* Gaud. Wimmera ryegrass: winter annual from South Australia.
(3) *L. multiflorum* Lam. Italian ryegrass: biennial from Northern Ireland.
L. perenne L. Perennial ryegrass: the following varieties were used:
(4) Irish: short-duration, from Northern Ireland.
(5) Kent: long-duration, from old pastures in Kent.
(6) Hunsballe: long-duration, bred at Holstebro, Denmark.
(7) S 24: long-duration, bred at Welsh Plant Breeding Station, Aberystwyth.
(8) S 101: long-duration, bred at Welsh Plant Breeding Station, Aberystwyth.

The inductive treatments comprised exposure of the germinating seed to low temperature (0–3° C.) in the refrigerator for 0, 4, and 8 weeks, followed by short day (8 hr.) in a warm greenhouse for 0, 4, and 8 weeks. In this experiment short days always *followed* the cold treatment. After the inductive treatments the material was transferred on 31 August 1956 to continuous light in a warm greenhouse. Supplementary lighting was supplied by 100-watt filament bulbs, giving an intensity at plant level of 30–150 ft. candles.

Eighty seedlings were recorded in each variety/treatment combination; leaf number on the main shoot was taken as the measure of rapidity of heading, and all seedlings which had not headed by the 25th leaf were recorded as non-flowering. In material transferred from short days, the leaf number after transfer to continuous light was used to give more accurate comparisons with the other treatments.

The inductive effect of short day alone (Table 1) was parallel to that of low temperature, but was not so great. In the summer and winter annuals, Westerwolds and Wimmera, all plants produced heads without short days or low temperature, but the winter-annual Wimmera showed a quantitative acceleration of heading after both these treatments. In the perennial varieties, most plants failed to head without either cold or short day, while the biennial Italian was intermediate in behaviour, 40 per cent. of the seedlings producing heads without any inductive treatment.

In the longer treatments, differences between the perennial varieties became apparent. The short-duration Irish ryegrass showed a smaller inductive requirement than the more persistent varieties, and these differed among themselves. Even so, considerable genetic variation existed within each variety. In Wimmera, for instance, the leaf number in the untreated material varied from 6 to 18, but after 8 weeks of cold treatment, all plants were reduced to 4 or 5 leaves. Similarly in Irish ryegrass, a few plants could head without any inductive treatment, but others required more than 8 weeks of exposure to cold.

When the interaction of low temperature and subsequent short day is considered, the picture becomes more complex. Material which was fully induced, either genetically, as in Westerwolds, or by low temperature alone, as in Wimmera after 8 weeks of cold treatment, showed no further acceleration by short day. Where induction was not complete, the short day tended to have an additional inductive effect.

TABLE I

Effect of Low-temperature and Short-day Induction on Populations of Lolium

All transferred to continuous light on 31 August 1956

		Cold treatments								
		None			4 weeks			8 weeks		
		Short day (weeks)			Short day (weeks)			Short day (weeks)		
		None	4	8	None	4	8	None	4	8
Westerwolds	% heading*	100	100	100	100	100	100	100	100	100
	Leaf no. Mean	4·9	5·1	4·8	4·3	4·4	4·2	4·3	4·7	4·2
	Variance	0·36	0·67	0·91	0·18	0·55	1·38	0·26	0·57	1·05
Wimmera	% heading	100	100	100	100	100	100	100	100	100
	Leaf no. Mean	11·3	8·0	4·1	5·6	5·1	4·5	4·2	4·9	4·7
	Variance	6·45	8·11	1·18	1·59	1·48	1·05	0·20	1·81	0·81
Italian	% heading	40	46	65	57	56	78	96	75	75
	Median leaf no.	—	—	8·6	12·1	8·5	6·7	5·8	5·3	5·6
Irish	% heading	12	26	27	19	48	36	81	78	74
	Median leaf no.	—	—	—	—	—	—	7·9	8·9	4·5
Kent	% heading	0	16	30	4	34	25	26	42	63
	Median leaf no.	—	—	—	—	—	—	—	—	9·0
Hunsballe	% heading	5	15	21	6	20	30	65	72	67
	Median leaf no.	—	—	—	—	—	—	9·8	8·5	6·9
S 24	% heading	0	6	13	0	12	40	34	39	42
	Median leaf no.	—	—	—	—	—	—	—	—	—
S 101	% heading	0	0	8	0	15	14	13	46	56
	Median leaf no.	—	—	—	—	—	—	—	—	8·4

* Transformed to degrees for analysis.

For leaf number, significant difference at $P = 0{\cdot}05$ is 0·72.

For leaf number, significant difference at $P = 0{\cdot}01$ is 1·09.

For % heading, significant difference at $P = 0{\cdot}05$ is 13·6° representing 5·5% at 0 and 100% and 23·4% at 50% level.

For % heading, significant difference at $P = 0{\cdot}01$ is 20·5° representing 12·5% at 0 and 100% and 35·0% at 50% level.

The results from this first experiment suggest that both short day and low temperature independently have an inductive effect, that the inductive requirement differs with the variety, and that considerable genetic variation in response occurs within each of these outbreeding populations.

Spring sown plants 1958. In this experiment the inductive treatments were increased to 12 weeks in the hope of obtaining complete induction; and short day and low temperature were provided simultaneously in the refrigerator.

The same outbreeding populations of *Lolium* were used, except that S 101 was replaced by New Zealand permanent pasture ryegrass, a short-duration perennial; the following single lines of wheat and the self-fertilizing *L. temulentum* were also included:

Wheat

Fylgia, spring variety from Sweden.
Bersee, alternative variety from France.

Squarehead's Master, winter variety from England.
Victor, winter variety from England.

These lines were obtained from the Botanic Garden of the Department of Agricultural Botany, University College of Wales, Aberystwyth.

TABLE 2

Effect of Low-temperature and Short-day Induction on Populations of Lolium

All transferred to continuous light on 30 January 1958

Population			No induction	Short days only (weeks)			Low temperature and short days (weeks)			Low temperature only (weeks)		
				4	8	12	4	8	12	4	8	12
Westerwolds	% heading*		100	100	100	100	100	100	100	100	100	100
	Leaf no.	Mean or median	4·5	5·1	5·0	5·0	4·7	4·8	5·1	4·9	5·1	5·1
		Variance	0·28	0·52	0·45	0·89	0·17	0·17	0·17	0·28	0·34	0·56
Wimmera	% heading		100	100	100	100	100	100	100	100	100	100
	Leaf no.	Mean or median	10·7	5·1	4·6	4·6	5·4	4·8	4·3	4·7	5·0	5·0
		Variance	17·97	0·54	0·39	0·68	1·30	0·25	0·27	0·49	0·45	0·50
Italian	% heading		57	93	100	100	83	100	100	72	94	100
	Leaf no.	Mean or median	16·3	9·1	5·8	5·0	12·5	8·2	7·3	14·3	11·9	8·4
		Variance	—	—	1·67	1·13	—	2·72	2·16	—	—	4·45
Irish	% heading		42	90	100	100	48	97	100	57	95	100
	Leaf no.	Mean or median	—	7·8	4·6	4·1	—	8·0	8·5	16·3	10·0	10·0
		Variance	—	—	0·57	1·45	—	—	3·13	—	—	5·09
New Zealand	% heading		66	89	100	100	70	100	100	66	92	100
	Leaf no.	Mean or median	13·0	9·0	4·9	4·8	14·3	10·1	10·4	13·6	10·0	10·3
		Variance	—	—	1·30	1·86	—	3·50	5·40	—	—	2·78
Kent	% heading		8	84	100	100	13	95	100	48	82	100
	Leaf no.	Mean or median	—	9·4	5·3	5·0	—	9·2	9·3	—	11·4	11·6
		Variance	—	—	1·42	1·34	—	—	2·65	—	—	5·73
Hunsballe	% heading		20	55	100	100	35	65	90	34	52	85
	Leaf no.	Mean or median	—	16·0	6·0	4·9	—	14·0	9·9	—	15·0	10·7
		Variance	—	—	2·73	2·16	—	—	—	—	—	—
S 24	% heading		27	55	100	100	35	49	55	33	63	86
	Leaf no.	Mean or median	—	16·1	6·9	5·9	—	—	15·5	—	13·0	11·9
		Variance	—	—	11·4	11·6	—	—	—	—	—	—

* Transformed to degrees for analysis.
For leaf number, significant difference at $P = 0·05$ is 0·94.
For leaf number, significant difference at $P = 0·01$ is 1·25.
For % heading, significant difference at $P = 0·05$ is 21·4° representing 13·3% at 0 and 100% and 36·4% at the 50% level.
For % heading, significant difference at $P = 0·01$ is 28·7° representing 23·0% at 0 and 100% and 48·0% at the 50% level.

L. temulentum

Ba 3081. Summer annual from Aberystwyth.
Ba 6137 (21). Moderate winter annual from Tekirdag, Turkey.
Ba 6139 (7). Moderate winter annual from Manyas, Turkey.
Ba 6902. Extreme winter annual from Versailles, France.

The three treatments were: (*a*) short day (8 hr.) in a warm greenhouse; (*b*) low temperature induction only, as in the previous experiment; and (*c*) low temperature and short day given simultaneously to young seedlings at the first leaf stage; the short-day illumination was provided by 15-watt daylight fluorescent tubes inside the refrigerator.

Each inductive treatment was given for 0, 4, 8, and 12 weeks and all the material was transferred on 30 January 1958 to continuous light in a warm greenhouse. Forty seedlings of each outbreeding population and ten of each single line were recorded for each variety/treatment combination.

The results for the outbreeding populations are shown in Table 2, and for the self-fertilizing lines in Table 3. The response to short-day induction alone was much greater than in the previous experiment. The annuals again showed no obligatory requirement for short day or low temperature, but most plants of the perennial varieties failed to head without cold or short days. Four weeks of short-day induction had a large effect, and all the material produced heads after 8 weeks of treatment. The short-duration perennials and Kent ryegrass appeared to be fully induced after 8 weeks of short days, but in S 24 and Hunsballe induction was only completed after 12 weeks. The New Zealand variety was very similar to the Irish in behaviour, and easily distinguishable from S 24, which it resembles in certain agronomic characters.

TABLE 3

Effect of Low-temperature and Short-day Induction on Lines of Wheat and Lolium temulentum

All transferred to continuous light on 30 January 1958

			No induction	Short days (weeks)			Low temperature and short days (weeks)			Low temperature (weeks)		
				4	8	12	4	8	12	4	8	12
Wheat												
Fylgia	Leaf no.	Mean	6·0	5·7	5·5	4·0	6·0	6·0	6·1	6·3	6·5	6·3
		Variance	0·00	0·15	0·20	0·24	0·00	0·00	0·10	0·15	0·26	0·15
Bersee	Leaf no.	Mean	7·9	6·2	5·6	4·7	6·5	6·2	6·3	7·0	7·0	Dead
		Variance	0·30	0·25	0·15	0·55	0·30	0·20	0·25	0·00	0·00	
Squarehead's Master	Leaf no.	Mean	18·0	9·3	6·4	5·5	9·9	7·7	7·1	10·1	7·9	7·8
		Variance	0·70	0·35	0·30	0·00	0·10	0·15	0·10	0·54	0·35	0·15
Victor	Leaf no.	Mean	20·0	10·7	6·5	5·9	11·7	8·0	7·3	11·6	7·9	7·9
		Variance	1·11	0·38	0·10	0·55	0·45	0·00	0·25	0·93	0·10	0·10
L. temulentum												
Ba 3081	Leaf no.	Mean	5·0	4·7	5·2	4·5	5·0	5·0	5·0	5·0	5·1	5·1
		Variance	0·00	0·15	0·20	0·26	0·00	0·00	0·00	0·00	0·10	0·10
Ba 6137 (21)	Leaf no.	Mean	12·7	5·6	6·0	4·5	6·3	5·8	6·0	5·9	5·8	5·9
		Variance	0·40	0·20	0·25	0·00	0·25	0·20	0·00	0·10	0·18	0·10
Ba 6139 (7)	Leaf no.	Mean	9·7	5·3	4·4	4·8	6·0	5·1	5·0	5·1	5·3	5·7
		Variance	0·45	0·15	0·10	0·25	0·00	0·10	0·00	0·10	0·23	0·26
Ba 6902	Leaf no.	Mean	19·2	14·1	4·5	Dead	13·3	6·0	6·0	9·6	6·1	6·0
		Variance	0·56	0·75	0·41		2·5	0·00	0·00	4·25	0·10	0·00

For leaf number, significant difference at $P = 0·05$ is 0·57.
For leaf number, significant difference at $P = 0·01$ is 0·76.

The effects of cold induction alone were similar to those in the previous experiment. Most plants of perennial ryegrass showed full induction after 8 weeks of cold treatment but varietal differences were again apparent, Hunsballe and S 24 showing the greatest requirement.

The combination of cold and short day in the refrigerator gave no greater inductive effect than did low temperature alone, suggesting that at these low temperatures short-day induction does not occur. The survival and vigour of the seedlings in this treatment were much better than in low temperature alone.

In the self-fertilizing lines of wheat and *L. temulentum* all plants headed without cold or short days. The summer annuals showed little or no response to induction and headed rapidly under all treatments. The winter annuals gave a parallel quantitative response to either cold or short day alone, and as with the outbreeders, short day in the refrigerator had no further inductive effect.

Considerable variation in response was again noticeable within each outbreeding population; in Irish, for instance, 42 per cent. of the plants produced heads without cold or short day, but 5 per cent. required more than 8 weeks of induction. By contrast, little or no genetic variation occurred within any of the self-fertilizing lines. All the plants produced heads and there was rarely a difference of more than one leaf number within a single treatment.

These results show that in the present populations of *Lolium*, floral induction can be brought about by short day or low temperature independently, but short day has no effect at temperatures of 0–3° C. Varietal differences in response to short day are parallel to those to low temperature, and appear to be related to the annual or perennial habit of the material. There is considerable range in inductive requirement within each outbreeding population, and rapid response to selection might be expected. No such genetic variation is apparent within any of the single lines of the self-fertilizing wheat or *L. temulentum*.

Although the relative order of the populations remained the same in each experiment, there were marked differences in the exact percentages of heading plants and the leaf numbers before heading in the two experiments. Two possible reasons for this are: (i) differences in the temperature during short-day induction; although the greenhouse minimum rarely went below 50° F. in either case, the mean temperature differed in the two experiments; and (ii) differences in temperature in the greenhouse under continuous light, which might well affect the degree of devernalization. These temperature effects are being studied in greater detail.

MEDITERRANEAN POPULATIONS

Further information on the relation between inductive requirement and the annual or perennial habit has been obtained from several Mediterranean populations of *L. rigidum* and *L. perenne*, ranging from Israel and Greece in the east to Morocco and Portugal in the west. These were obtained from the Joint FAO/CSIRO Collection by courtesy of Mr. Ch. Rossetti, FAO, Rome. Details of the original habitats have been published by FAO (1955) and morphological descriptions are given by Rossetti (1958).

Thirty-two populations of *L. rigidum* and 11 of *L. perenne* were given 0, 4, and 8 weeks of induction by low temperature as in the previous experiment, and transferred to continuous light in a warm greenhouse on 24 January 1957. Wimmera ryegrass (*L. rigidum*) from South Australia, and Irish perennial ryegrass were included as controls.

This Mediterranean material (Table 4) falls into two distinct flowering groups, corresponding to the annual (*L. rigidum*) and the perennial (*L. perenne*) populations. In *L. rigidum*, nearly all plants produced heads rapidly without any cold treatment, although there was a slight quantitative response in all samples, and after 8 weeks of exposure many seedlings headed with only three leaves on the main shoot. No relation could be traced between the rapidity of heading and the latitude, altitude, or rainfall of the original habitat. Wimmera ryegrass from South Australia showed similar behaviour to the Mediterranean samples of *L. rigidum*, but had a slightly higher leaf number when grown without cold treatment.

TABLE 4

Response to Low-temperature Induction in Mediterranean Populations of Lolium

Leaf number before heading

		Cold treatment (weeks)						
		8		4		None		
Origin	No. of samples	Mean of means	Mean variance	Mean of means	Mean variance	Mean of means	Mean variance	Range
L. rigidum								
Egypt	1	3·2	0·17	3·7	0·24	4·6	1·22	3–7
Cyprus	1	3·3	0·20	Not tested		6·0	2·22	4–9
Israel	1	3·3	0·22	4·6	0·46	7·0	5·29	4–12
Cyrenaica	3	3·2	0·29	3·7	0·49	5·2	2·36	3–11
Algeria	8	3·5	0·20	4·2	0·46	5·8	2·38	3–19
Morocco	12	3·5	0·26	3·8	0·31	4·8	1·39	3–16
Portugal	3	3·5	0·26	4·3	1·10	5·8	2·70	3–13
Greece	1	3·7	0·33	5·3	2·13	8·1	7·24	5–14
Iran	1	3·4	0·31	4·4	1·74	7·5	4·58	4–13
S. Australia	1	3·9	0·34	5·8	2·89	9·4	5·95	5–15
		Mean of medians	Mean variance	Mean of medians	Mean variance	Mean of medians	Mean variance	Range
L. perenne								
Cyrenaica	1	8·0	97%	10·0	92%	11·5	79%	6–
Algeria	9	9·7	96%	12·2	82%	14·8	71%	4–
Italy	1	9·5	100%	11·5	79%	10·5	78%	7–
N. Ireland	1	9·2	75%	—	43%	—	15%	12–

The Mediterranean populations of *L. perenne* were quite distinct. They all contained some plants (from 4 to 54 per cent.) with an obligatory requirement for low temperature, but also some which headed rapidly without any cold treatment. By contrast, 85 per cent. of the plants of Irish perennial ryegrass possessed an obligatory cold requirement and 25 per cent. needed more than 8 weeks of induction.

These Mediterranean populations of *L. perenne* are evidently intermediate in their inductive requirements between the typically annual *L. rigidum* and the North-European varieties of *L. perenne*, but even so the results show a clear relation between the annual or perennial habit and the inductive requirement. The annual *L. rigidum* has no obligatory cold requirement, while at least some plants in each population of *L. perenne* fail to head without cold treatment.

RESPONSE TO SELECTION

The above results indicate that considerable genetic variation in inductive requirement exists within each outbreeding population, and that rapid response to selection should be possible. The response to selection for high and low

TABLE 5

Selection for Inductive Requirement. Wimmera Ryegrass

(i) *Sown in continuous light in greenhouse 2 September 1955 without induction. (Leaf number before heading)*

Original population	Mean Variance	10·9 10·5			
1st generation	Mean Variance	8·8 8·8		13·5 9·9	
2nd generation	Mean Variance	8·4 5·3	13·0 11·1	10·4 6·5	12·8 9·2
3rd generation	Mean Variance	8·2 2·1	15·4 9·3	10·1 3·7	12·9 5·7
		Low–low	Low–high	High–low	High–high

Significant difference at $P = 0·05$ is 1·26.
Significant difference at $P = 0·01$ is 1·79.

(ii) *Sown outdoors 3 March 1955. (Date of ear emergence)*

Original population	Mean Variance	26·7 June 538·7			
1st generation	Mean Variance	11·8 June 242·6		9·3 July 283·6	
2nd generation	Mean (or median) Variance	9·8 June 37·4	27·4 June 215·6	19·8 June 124·7	25·5 July (86% headed)
3rd generation	Mean (or median) Variance	8·4 June 60·9	24·1 June 208·0	24·4 June 148·5	27·0 July (78% headed)
		Low–low	Low–high	High–low	High–high

Significant difference at $P = 0·05$ is 8·4 days.
Significant difference at $P = 0·01$ is 11·8 days.

(iii) *Correlation between greenhouse and field means* = +0·656* ($n = 9$)

(iv) *Correlation between autumn-sown* (23 August 1954) *and spring-sown* (3 March 1955) means = $+0·449^{NS}$ ($n = 9$)

inductive requirement was therefore studied in two experiments, one using Wimmera ryegrass, and the other Irish and Kent perennial ryegrass.

Wimmera ryegrass. In this variety there is no absolute requirement for either cold or short days and quantitative selection can be made on seedlings grown without any inductive treatment.

Fifty seedlings of Wimmera ryegrass were grown in continuous light in a warm greenhouse in September 1952, conditions under which rapidity of heading is a measure of inductive response. The four earliest and four latest heading plants were selected; the four earliest were allowed to interpollinate, and early and late lines established from them; and similarly, early and late lines were derived from the latest four parents. Details of this fourway method of selection have been given elsewhere (Cooper, 1959*b*). Selection in each generation was carried out under continuous light in a warm greenhouse, using supplementary filament lighting of an intensity of 30–150 ft. candles.

By spring 1955 the original population and lines from three generations of selection were available. These were tested for inductive requirements (i) under continuous light in a warm greenhouse, sown 2 September 1955, and (ii) spring-sown outdoors on 3 March 1955 and grown under natural photoperiods. In this way, it was hoped to see how far selection under greenhouse conditions had changed the winter requirement, as measured by the extent of heading after a spring sowing. This material was also sown outdoors in autumn 1954 to detect any correlation between heading date after an autumn and a spring sowing (Table 5).

The greenhouse results show that selection has been effective in all lines, the mean values for the extreme early and late lines differing by 4·7 leaves before heading. There is still appreciable genetic variation within each selection line in the third generation, indicating heterozygosity of many of the parents.

The field sowings present a similar picture. The early lines are considerably earlier than the original population and have smaller variances; the late lines are later, their variances are higher, and some plants in the last two generations of the latest lines failed to head.

The correlation between the spring-sown and greenhouse data is significant (+0·656) but only at the 5 per cent. level; the gradual change in natural photoperiod outdoors may well have differential effects on the rate of inflorescence development as compared with that of continuous light in the greenhouse. Selection for high or low inductive requirement in the greenhouse has evidently been reflected in the rate and extent of heading after a spring sowing in the field. There has, however, been no corresponding change in the date of ear emergence after an autumn sowing, suggesting that the inductive requirement for short day and/or low temperature, and the critical photoperiod for floral initiation and subsequent development, are under independent genetic control.

Irish and Kent perennial ryegrass. In these perennial populations most plants have an absolute requirement for cold or short days, and to select for quantitative differences the plants must be grown under partially inductive treatments. In summer 1953 seedlings of Irish and Kent ryegrass were given 0, 6, and 10 weeks of low-temperature induction, and transferred to continuous light in a warm greenhouse on 2 September 1953. Forty seedlings were recorded in each treatment.

In Irish ryegrass very few plants headed without low temperature, but 40 per cent. did so after 6 weeks of cold treatment, and the four earliest of these were intercrossed as the basis of the low requirement line. Conversely, in the 10 weeks' treatment 75 per cent. of the plants produced heads, and the latest four of these were intercrossed to form the high requirement line.

TABLE 6

Selection for Inductive Requirement. Irish and Kent Ryegrasses

(*Percentage of heading plants*)

(i) *Sown outdoors 30 January 1957*

	Irish				Kent			
Original population	58				35			
1st generation	98		56		72		14	
2nd generation	94	69	88	48	100	66	35	7
	Low–low	Low–high	High–low	High–high	Low–low	Low–high	High–low	High–high

Significant difference at P = 0·05 is 26·9° representing 20·4% at 0% and 100%, and 45·4% at the 50% level.
Significant difference at P = 0·01 is 40·8° representing 42·7% at 0% and 100%, and 65·4% at the 50% level.

(ii) *Four weeks of cold treatment, then 4 weeks of short day and transferred to continuous light on 26 September 1956*

	Irish				Kent			
Original population	49				20			
1st generation	80		28		73		14	
2nd generation	86	Not tested	73	31	93	Not tested	Not tested	11
	Low–low	Low–high	High–low	High–high	Low–low	Low–high	High–low	High–high

Significant difference at P = 0·05 is 21·2° representing 13·1% at 0% and 100%, and 36·0% at the 50% level.
Significant difference at P = 0·01 is 32·1° representing 28·2% at 0% and 100%, and 53·0% at the 50% level.

(iii) *Correlations between percentage of heading plants in different tests*

	r	*n*
Outdoors, sown 3 March 1955		
×Outdoors, sown 30 January 1957	+0·715***	17
×Greenhouse (4 weeks cold+4 weeks short day)	+0·684***	18
Outdoors, sown 30 January 1957		
×Greenhouse (4 weeks cold+4 weeks short day)	+0·718***	41

Similarly, in Kent ryegrass, no plants produced heads without cold, but the earliest four to head after 6 weeks of induction formed the basis of the low line, and the latest four after 10 weeks of treatment gave rise to the high line.

In the next generation, a high and low line were derived from each initial group of parents, but the criterion of selection was modified, the partially inductive treatments being provided by two sowing dates in the spring (7 January and 3 March 1955).

In the January sowing nearly all plants headed, and the high-requirement parents were selected from the latest of these. In the March sowing, only a few plants headed and low-requirement parents could be selected from the earliest of these.

In 1956–7 the original populations and the first two generations of selection were tested for inductive requirements in two ways; (i) sown outdoors 30 January 1957, and (ii) given 4 weeks' cold treatment (0–3° C.) followed by 4 weeks of short day (8 hr.), then transferred to continuous light in a warm greenhouse on 16 August 1956. In the field tests, the number of plants in head at weekly intervals throughout the summer and the final number in head by the end of September were recorded, while in the greenhouse tests, leaf number before heading and the final number of heading plants were taken (Table 6).

The field and greenhouse tests show that selection has been effective in both varieties, though rather more so in Kent, in which a range from 100 to 7 per cent. of heading plants has been obtained in the second generation of selection outdoors. The character recorded—final percentage of heading plants—is an all-or-nothing response, and so no direct measure of the variance of the original population or of the selection lines is possible, but the results from the second generation of selection suggest that appreciable genetic variation can be released from within four plants of similar phenotype.

The correlations between the greenhouse results and each spring sowing are high (+0·718 and +0·684) and of the same order as the correlation between the two spring sowings themselves (+0·715). This suggests that the response to standard inductive treatments in the greenhouse gives a good measure of winter requirement in the field.

There was again no correlated change in the date of heading after an autumn sowing, suggesting independent genetic control of inductive requirements and critical photoperiod for floral initiation.

It is clear from the above results that in Wimmera ryegrass and in Irish and Kent perennial ryegrass, the requirement for short-day and low-temperature induction is under genetic control, that considerable genetic variation exists between and within these outbreeding populations, and that rapid response to selection can occur.

DISCUSSION

The present work throws light on the physiology of flowering in *Lolium* compared with other Gramineae, and gives information about the genetic structure of these outbreeding species. It also paves the way for the establishment of standard greenhouse techniques for the rapid screening and analysis of populations (Cooper, 1960).

It appears that in *Lolium* either short day or low temperature can act as alternatives for floral induction, and that a continuous range in inductive requirement exists within the genus. By contrast, Wycherley (1952) reported that natural short days in a warm greenhouse had no inductive effect on clones of *L. perenne* and that cold was essential for floral induction in this species. These contrasting reports may be due to genetic differences in the material used, but the present results indicate that the temperature at which the short days are given affects the degree of induction, while devernalization by subsequent high temperature may also remove the inductive effect of short days. Further studies are needed on this point.

In other Gramineae also the inductive requirement differs with the species and population. The most detailed studies on low-temperature and short-day induction are those on Petkus winter and spring rye (Purvis and Gregory, 1937; Gregory, 1948; Gott, Gregory, and Purvis, 1955). Spring rye has no inductive requirement for either cold or short day and will produce heads under continuous light in a warm greenhouse after a leaf number of about 6. It thus corresponds to Westerwolds ryegrass and to the summer-annual lines of wheat and of *L. temulentum* used in the present work. Winter rye, on the other hand, produces heads without induction only after a leaf number of 13–17 in continuous light, or over 20 in natural long days. It can, however, be fully induced by about 8 weeks of cold treatment and then heads as rapidly as the spring variety. Short days (8 or 10 hr.) given during the first 6 to 8 weeks of growth also have a definite inductive effect on unvernalized winter rye, both the rate of apex elongation and spikelet initiation being more rapid in short days than in long days. Winter rye thus corresponds to the winter-annual lines of wheat and *L. temulentum*, and to the more extreme winter-annual plants of Wimmera ryegrass. Similar alternative induction by cold or short day has been reported for other winter cereals (Gregory, 1948) and may be general for winter annuals.

In perennial forage grasses, the inductive requirements are usually more extreme. Gardner and Loomis (1953) basing their results on the flowering behaviour after a spring sowing in Iowa, U.S.A. reported a definite winter requirement for several north temperate perennial species including *Dactylis glomerata*, *Poa pratensis*, and *Lolium perenne*, but no such winter requirement was shown by the north temperate species *Arrhenatherum elatius* and *Phleum pratensis*, nor by a number of range grasses.

In some of these species, the physiological basis of floral induction has been studied in more detail. In *Poa pratensis*, Peterson and Loomis (1949) reported that both short days and low temperatures are necessary for floral induction, and similar requirements have been found by Newell (1951) in *Bromus inermis*. In *Dactylis glomerata*, however, Gardner and Loomis (1953) state that while both short days and cold are necessary, they can be given separately, provided the short days precede the low temperature. In their material a week of warm, long days could destroy the short-day inductive effect. Wycherley (1952) also found that both cold and short day were necessary in *Dactylis* and that induction was not possible by cold alone.

In most of the above studies, marked differences in inductive requirement between genotypes were recorded, and it is clear that considerable variation occurs both between and within species in the relative roles of low temperature and short day in floral induction.

The existence of large genetic differences in inductive requirements within the same or closely related species is emphasized by the present results. In *Lolium*, the differences between populations are clearly related to past climatic and agronomic selection. Westerwolds ryegrass which has been selected as a summer-annual catch crop, shows no inductive requirement, while in the perennial varieties an obligatory winter requirement prevents the tillers formed during mid- to late-summer from flowering, and ensures an overlapping succession of vegetative tillers from year to year.

There is, however, no inevitable correlation between the requirements for short day and/or low temperature before floral induction, and the critical photoperiod for floral initiation and subsequent elongation. As the present selection results indicate, these two stages of inflorescence development appear to be under independent genetic control, and have been selected independently in the past. The biennial Italian ryegrass, for instance, is intermediate between the early- and late-flowering perennials in date of heading but has considerably smaller requirements for cold or short-day induction; while the three early-flowering varieties of perennial ryegrass, Irish, New Zealand, and S 24, have a similar date of ear emergence in the spring but differ considerably in inductive requirement. The relation between flowering requirements and past selection history in Wimmera, Irish, and Kent ryegrass has been discussed in more detail elsewhere (Cooper, 1959*a*).

Each outbreeding population still contains appreciable genetic variation between individuals in inductive requirements, and this results in a rapid response to any change in the direction or intensity of selection. In Kent ryegrass, for instance, two generations of selection have produced contrasting lines with 7 and 100 per cent. of heading plants after a particular spring sowing.

Furthermore, in this outbreeding material, most plants are heterozygous for genes controlling inductive requirements and appreciable genetic variation is released in each generation by segregation and recombination, providing a further important source of variation for long-term response to selection.

Such a genetic structure, allowing of close adaptation to the average selective background, but at the same time considerable storage of genetic variation, is typical of outbreeding populations (Mather, 1953) and has already been described for date of ear emergence in *Lolium* (Cooper, 1959 *a*, *b*). It is evident that the inductive requirements in this genus provide yet another example of this type of genetic control.

SUMMARY

1. The response to short day and low-temperature induction has been studied in a number of outbreeding populations of *Lolium perenne*, *L. multiflorum*, and *L. rigidum* from different climatic and agronomic origins.

2. Floral induction can be brought about by either low temperature

(0–3° C.) or short day (8 hr.) independently, but short day is ineffective at low temperatures. Short days following low temperature have an additional effect on partially induced material, but the temperature at which the short days are given is also important.

3. The inductive requirement varies with the climatic or agronomic origin of the population. The summer-annual Westerwolds ryegrass needs neither short days nor cold, while the winter-annual Wimmera ryegrass shows a quantitative response to these factors. Most plants of perennial ryegrass from temperate latitudes have an obligatory requirement for either cold or short days before floral induction. This is greatest for the long-duration varieties, such as Kent and S 101, and lower for the short-duration varieties such as Irish and New Zealand perennial ryegrass. The biennial Italian ryegrass is intermediate, some 50 per cent. of the plants showing an obligatory requirement for induction, and the remainder a quantitative response.

4. The analysis of a number of Mediterranean populations of *L. rigidum* and *L. perenne* confirmed this relation between the inductive requirement and the annual or perennial habit, but the Mediterranean populations of *L. perenne* were intermediate in their inductive requirements between the annual *L. rigidum* and north European varieties of *L. perenne*.

5. Considerable genetic variation could be detected within each outbreeding population, most plants proved to be heterozygous for genes controlling inductive requirement, and rapid response to selection was possible in Wimmera, Irish, and Kent ryegrass. No such genetic variation was apparent within single lines of the self-fertilizing *L. temulentum*.

6. There was a good correlation between the inductive requirement as assessed from standard cold and short-day treatments, and the winter requirement as measured by the extent of heading after a spring sowing outdoors.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the interest shown and facilities provided by Professor E. T. Jones, former Director, and Professor P. T. Thomas, present Director of the Welsh Plant Breeding Station. My thanks are due to Dr. E. L. Breese and Dr. Martin Borrill for helpful discussion and I am also grateful to Miss Irene D. Rees for valuable assistance with the manuscript.

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Effects of Nitrogen Nutrition on Flowering in Carnation

BY

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ABSTRACT

Two varieties of perpetual flowering carnation were grown under various conditions of nitrogen supply, daylength, and temperature.

In short days as compared with long, the number of leaves below the flower bud was always greater and the flower bud always appeared later.

At low levels of nitrogen, successive leaf pairs developed more slowly and more leaves were formed below the flower, so that flower initiation was delayed. This effect was more marked under short days than under long days.

The development of the flower bud from macroscopic appearance to anthesis was affected primarily by temperature. Low temperatures generally retarded development, this effect being most pronounced in nitrogen deficient plants grown under short days.

INTRODUCTION

IN reviews on the physiology of flowering there has been a divergence of opinion on the role of inorganic nutrition in floral initiation. Leopold (1951) has suggested that inorganic nutrients can affect rate of flower development but that they have little or no effect on floral initiation. Doorenbos and Wellensiek (1959) have recently given support to this view but admit an exception in an effect of nitrogen nutrition on flower initiation in *Sinapis alba*. Melchers (1952), on the other hand, has suggested that nitrogen nutrition can affect the time of floral initiation in a number of plants.

The experiments described in this paper were undertaken by one of us (J. B.) to examine the influence of inorganic nutrients on initiation and development of flowers in the perpetual flowering carnation. The plant forms a terminal flower so that it has been possible to use the number of leaves to flower (Purvis, 1934; Purvis and Gregory, 1937) as a criterion of effects of treatment on flower initiation. The carnation has no absolute photoperiodic requirement for flower initiation (Post, 1942) but it has been shown that initiation is more rapid under long than under short days (Blake, 1955). Experiments were therefore designed to expose possible interactions between effects of day length and nutritional treatment on flowering. Some of the results to be reported here have been communicated in condensed form to the International Horticultural Congress (Blake, 1955).

MATERIALS AND METHODS

The results to be reported are from three glasshouse experiments. In each of the first two experiments two varieties of carnation, Maytime and Puritan, were used; in the third Puritan was used alone.

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

Cuttings were taken from stock plants grown under natural-day conditions and were treated with a rooting hormone, β-indolylbutyric acid at a concentration of 500 p.p.m. in 50 per cent. ethanol. After rooting they were planted in silica sand either one to a $3\frac{1}{2}$-inch pot (Expt. 1 and 2) or four to a container ($9 \times 8 \times 4\frac{3}{4}$ in.), lined with bituminous paint (Expt. 3). The plants were allowed to become established for a period of 2 to 4 weeks during which time they received a complete nutrient solution. Plants of any one variety within an experiment were then stopped back to a constant number of leaves. Breaks from the axillary buds were allowed to run up to flower without subsequent stopping. In Expt. 3 the number of breaks allowed to flower was limited to two, the remainder being removed at an early stage.

The three experiments were conducted in different years but in each experiment cuttings were taken in November or December and treatments were commenced in January or February.

Nutrient supply

The standard nutrient solution employed had the following composition:

Element	*Conc. p.p.m.*	*Source*
N	150	$Ca(NO_3)_2 \cdot 4H_2O$
Ca	215	$Ca(NO_3)_2 \cdot 4H_2O$
P	60	H_3PO_4 (Sp. Gr. 1·75)
K	150	K_2SO_4
Mg	50	$MgSO_4 \cdot 7H_2O$
S	129	$MgSO_4 \cdot 7H_2O, K_2SO_4$
Fe	8	Ferric citrate
Mn	1	$MnSO_4 \cdot 4H_2O$
B	1	H_3BO_3

The solution was made up with tap water and the pH was adjusted to 5·6–5·8 with sulphuric acid.

In Expt. 1 and 2 nutrient solution was applied to the surface of the sand in the pots at intervals of 4 to 10 days depending on the season of the year. Water was given as necessary during the intervening periods. In Expt. 3 plants were supplied with nutrient solution by sub-irrigation at 36- to 48-hour intervals. In this experiment ferric sodium ethylene-diamine tetra-acetate supplying 5 p.p.m. Fe was substituted for ferric citrate in order to avoid precipitation in the reservoirs of nutrient solution.

Daylength treatments

All plants received a basic short-day of 8 hours of natural daylight. This constituted the short-day treatment. For the long-day treatment, supplementary illumination was given for $4\frac{1}{2}$ hours at either end of the period of natural daylength, thus extending the length of day to 17 hours. The intensity of the supplementary illumination was about 1,000 lumens per sq. m. in Expt. 1 and 200–600 lumens per sq. m. in Expt. 2 and 3. The light sources were 'daylight' fluorescent tubes which in Expt. 3 were supplemented with incandescent lamps.

Temperature treatments

In Expt. 2 and 3 plants were grown at two temperatures. In the high-temperature treatment the temperature was always maintained above 15° C. (60° F.). For the low-temperature treatment night temperatures were allowed to drop to *c.* 10–12° C. (50–55° F.), the higher temperature obtaining during the summer months. There was little difference in the maximum temperatures attained during the daytime in these two treatments. The night temperature in Expt. 1 was equivalent to that of the low-temperature treatments of Expt. 2 and 3.

Assessment of results and experimental layout

Data collected included the number of weeks to the appearance of the first flower bud on each plant and the number of pairs of leaves, including floral bracts, on the first shoot to produce a flower bud. Flower buds could be identified at an early stage by the appearance of floral bracts. These were usually four in number, broader and shorter than the leaves, and with an acuminate tip. Records were also kept of the number of days from the appearance of the flower bud to full expansion of the petals ('anthesis'). The length of the stem was measured at this stage, and from this and the number of leaf pairs the mean internode length at anthesis was calculated.

Facilities did not allow replication of daylength or of daylength-temperature treatments. In Expt. 1, there was a time interval between the planting of the two varieties of carnations and they were grown separately during the course of the experiment. Within each daylength treatment each nutritional treatment was replicated in three plots of six pots each. In Expt. 2, within each daylength-temperature treatment, each variety was replicated in two plots of six pots each. These plots were further split into two sub-plots with three pots at each level of nitrogen. In Expt. 3, under each daylength-temperature treatment, each nutritional treatment was replicated in four plots (each of a single container) of four plants each. In each experiment the plots representative of the nutritional treatments were randomly distributed within the daylength or daylength-temperature treatments. Significant differences presented in the tables have been calculated by analysis of variance. Since all the experimental designs were of the 'split plot' type, with 'main treatments' unreplicated, the appropriate error variances could only be estimated for the nutritional treatments and for interactions of these with daylength and temperature. Average effects of daylength or temperature could not be tested statistically nor could the 2-factor interaction between them.

RESULTS

Experiment 1. The first experiment was designed to examine effects on flowering of low levels of nitrogen, phosphorus, and potassium. In the 'low

N', 'low P', and 'low K' treatments the concentration of the element in question was at one-tenth of that in the standard nutrient solution. In lowering the level of nitrogen the concentration of calcium was maintained constant by the addition of $CaCl_2$. The effect of doubling the concentrations of all the salts used in the standard nutrient solution was also tested in this experiment.

The nutrient treatments were given under long and short days and also under natural days which varied in length from 9 to 17 hours but were probably from 15 to 17 hours long during the period of flower induction.

The results shown in Table 1 confirm the importance of photoperiod in affecting flowering in carnations (Blake, 1955). Thus under long days or natural days as compared with short days, appearance of flower buds was hastened and number of leaves to flower was reduced. This was true for both Puritan and Maytime under all the nutrient treatments given and the effects were so large as to be unlikely to be due to positional effects for the unreplicated treatments.

TABLE 1

Effects of Inorganic Nutrition and Daylength on Flowering in Carnation, var. Maytime and Puritan. (Experiment 1.) Each Value is the Mean of Measurements made on 18 Plants

		Maytime			Puritan		
Feature measured	Nutrients	Short-day	Natural-day	Long-day	Short-day	Natural-day	Long-day
Weeks to appearance of first flower bud	Standard	18·5	16·3	15·5	28·5	17·2	17·9
	Standard ×2 conc.	18·3	16·4	15·1	27·3	17·0	17·9
	Low N	20·0	17·9	15·8	48·1	19·1	19·9
	Low P	18·8	16·2	15·8	31·3	17·4	19·0
	Low K	18·6	16·4	14·4	27·8	17·3	18·3
Leaf pairs to flower	Standard	18·4	16·5	15·4	25·5	17·3	17·0
	Standard ×2 conc.	18·5	16·8	15·9	24·5	17·2	17·2
	Low N	20·2	16·7	15·4	33·0	17·2	17·3
	Low P	19·0	16·5	16·0	26·0	17·3	17·2
	Low K	18·9	16·5	15·5	24·7	17·1	17·3

Values for significant differences between nutrient treatment means:

		$P = 0·05$	$P = 0·01$
Weeks to appearance of first flower bud:	Maytime	0·63	0·85
	Puritan	4·19	5·65
Leaf pairs to flower:	Maytime	0·67	0·91
	Puritan	2·06	2·78

The low N, P, and K treatments were such that they produced no severe limitation of the growth of the plants. In the low N treatments visual symptoms of nitrogen deficiency did not show until 3 months after the commencement of the treatments. The leaves which then developed were a somewhat paler green and were more erect than in the other treatments. Symptoms of potassium deficiency showed up later as a progressive yellowing of the leaves from the base of the plant upwards. No deficiency symptoms were observed in the low P treatment.

Table 1 shows that in both varieties, the low N treatment caused delays in the appearance of flower buds and increases in the number of leaves to flower. These effects were marked under short days but were small or non-existent under long days and natural days. So marked was the effect of combination of low-nitrogen and short-day conditions on some plants of Puritan that they remained vegetative for about 5 months longer than the corresponding plants receiving the standard nutrient solution. The only other nutrient treatment to produce an effect was low K which apparently hastened the appearance of flower buds in Maytime, under the long-day treatment only. In this case, however, there was no associated difference in the number of leaves to flower and Puritan was not similarly affected.

Experiments 2 and 3. These experiments were designed to test effects of different levels of nitrogen supplied to plants grown under long and short days at high and low temperatures.

Two levels of nitrogen were employed in Expt. 2 and three levels in Expt. 3. In Expt. 2 the 'high N' treatment was that in which standard nutrient solution (150 p.p.m. N) was employed while the 'low N' treatment was the same as in Expt. 1 (15 p.p.m. N). In Expt. 3 'low N', 'medium N', and 'high N' treatments were given corresponding to nitrogen concentrations of 10 p.p.m., 50 p.p.m., and 250 p.p.m. respectively. $CaCl_2$ was added to the low and medium N solutions to bring the concentration of calcium to that of the high N solution.

There was no consistent effect of daylength on total stem length, but mean internode length was in every case greater in long days than in short (Tables 2 and 3). The low-nitrogen treatment supported a reasonable amount of growth so that at the time of anthesis the nitrogen-deficient plants had attained a stem length similar to or greater than the plants receiving the high level of nitrogen. There was, however, evidence that development was retarded by the low-nitrogen treatment. This is shown by the ratio of the time to the appearance of the flower bud to the number of leaf pairs below the bud, which was appreciably higher in the low-nitrogen treatments. There was a small but significant reduction in mean internode length under low-nitrogen conditions in Expt. 3 but not in Expt. 2.

In these experiments (Tables 2 and 3) as in the previous one, long days as compared with short days accelerated the appearance of flower buds and reduced the number of leaves to flower. These effects were produced irrespective of other superimposed conditions. Low-nitrogen supply again delayed the appearance of flower buds and increased the number of leaves to flower. These effects of nitrogen supply were marked under short days but did not reach significance under long days, while effects of daylength were more pronounced under low as compared with medium or high N treatments. There was no difference between the medium and high N treatments used in Expt. 3 suggesting that the nitrogen requirement for flowering was satisfied by the medium level.

TABLE 2

Effects of Nitrogen Level, Temperature, and Daylength on Flowering and Growth of Carnation, var. Maytime and Puritan. (*Experiment* 2.) *Each Value is the Mean of Measurements made on 6 Plants*

Feature measured	Daylength	Temperature	Maytime		Puritan	
			Low N	High N	Low N	High N
Weeks to appearance of first flower bud	Short	Low	35·4	20·8	41·6	32·1
		High	32·1	22·1	38·9	32·2
	Long	Low	20·6	17·6	25·9	20·8
		High	18·8	16·3	23·8	20·9
Leaf pairs to flower	Short	Low	31·6	22·8	31·2	27·5
		High	31·0	24·8	34·5	29·1
	Long	Low	19·0	18·1	19·6	19·3
		High	22·3	20·6	23·8	22·8
Weeks from budding to anthesis	Short	Low	9·0	5·3	15·0	7·9
		High	6·7	5·1	7·0	6·6
	Long	Low	5·4	5·8	6·1	5·9
		High	4·8	4·7	4·6	4·7
Length of stem at anthesis (cm.)	Short	Low	93·5	73·0	101·5	75·5
		High	80·5	73·5	92·5	69·5
	Long	Low	80·0	77·0	76·5	79·5
		High	82·0	80·0	88·5	76·0
Mean internode length (cm.)	Short	Low	2·9	3·2	3·2	2·7
		High	2·6	2·9	2·6	2·3
	Long	Low	4·2	4·0	3·9	4·2
		High	3·6	3·6	3·7	3·3

Values for significant differences between nitrogen treatment means:

	$P = 0·05$	$P = 0·01$
Weeks to appearance of first flower bud	6·8	9·6
Leaf pairs to flower	4·6	6·4
Weeks from budding to anthesis	3·0	4·2
Length of stem at anthesis	15·6	21·9
Mean internode length—no significant differences.		

Effects of temperature were less consistent than those of daylength and nitrogen supply. Numbers of leaves to flower were less in almost all cases under low as compared with the high-temperature treatments. However, under long days, these lower leaf numbers were sometimes associated with a delay instead of an acceleration in the appearance of flower buds. Under short days the effect of temperature on time of appearance of flower buds varied between the different nitrogen treatments.

Further experiments in which night and day temperatures were varied independently have indicated that relatively low night temperatures given under short days with a full-nutrient treatment hastened floral initiation in the variety White Sim (Blake and Spencer, 1958).

TABLE 3

Effects of Nitrogen Level, Temperature, and Daylength on Flowering and Growth of Carnation, var. Puritan. (Experiment 3.) Each Value is the Mean of Measurements made on 12 Plants

Feature measured	Daylength	Temperature	Low N	Medium N	High N
Weeks to appearance of first flower bud	Short	Low	40·7	25·0	24·1
		High	45·1	24·1	24·6
	Long	Low	25·0	16·7	16·8
		High	17·3	15·2	14·7
Leaf pairs to flower	Short	Low	28·4	24·3	24·1
		High	43·0	28·6	29·6
	Long	Low	20·8	18·1	18·5
		High	21·5	21·4	21·5
Weeks from budding to anthesis	Short	Low	11·1	5·6	5·1
		High	5·0	4·7	4·6
	Long	Low	5·4	5·7	5·6
		High	4·5	4·4	4·4
Length of stem at anthesis (cm.)	Short	Low	93·1	86·7	84·5
		High	110·7	87·6	91·0
	Long	Low	80·9	87·7	87·1
		High	82·9	83·4	84·0
Mean internode length (cm.)	Short	Low	2·9	3·5	3·5
		High	2·6	3·0	3·1
	Long	Low	4·4	4·8	4·7
		High	3·9	3·9	4·0

Values for significant differences between nitrogen treatment means:

	$P = 0·05$	$P = 0·01$
Weeks to appearance of first flower bud	4·0	5·5
Leaf pairs to flower	3·4	4·7
Weeks from budding to anthesis	0·6	0·8
Length of stem at anthesis	6·6	8·9
Mean internode length	0·35	0·48

Records of the number of weeks from the appearance of flower buds to anthesis for Expt. 2 and 3 (Tables 2 and 3) show a consistent but sometimes small effect of the high temperature treatment in hastening flower development. Under the low-temperature treatment there was an interaction of low nitrogen and short days to produce a marked delay in anthesis in both Maytime and Puritan.

DISCUSSION

The experiments reported here have shown that nitrogen nutrition can affect the time at which floral initiation occurs in carnation. In low-nitrogen treatments successive leaf pairs developed more slowly while the final number of leaves produced below the flower initials was increased. Hence in these treatments floral initiation was delayed as also was the appearance of the flower buds.

In other plants where nitrogen deficiency has been found to delay the appearance of flower buds, time of floral initiation has frequently proved to be unaffected (Purvis, 1934; Neidle, 1939; Naylor, 1941; Withrow, 1945). Such results have led to claims that inorganic nutrients can affect time of flowering through effects on flower development but that 'they have very little or no influence on floral initiation' (Leopold, 1951). However, important exceptions to this are on record. von Denffer (1940) showed that in spring barley and wheat flowering was accelerated and leaf number was markedly reduced by low nitrogen supply. Cajlachjan (1944) showed that mustard flowered more rapidly with a low nitrogen supply and Hinnawy (1956) working with the same species claimed a small effect of nitrogen deficiency in hastening floral initiation. Scully *et al.* (1945) showed that low nitrogen supply caused flowers to be produced at lower nodes in two of four varieties of soybean studied.

Thus there are reports in the literature which show acceleration of flowering and reduced leaf numbers associated with low levels of nitrogen supply. In carnation, however, the opposite was the case and low-nitrogen treatments produced mean increases of up to 14 pairs of leaves before the laying down of floral primordia. Of the plants studied by von Denffer (1940) some showed delayed flowering with low nitrogen but only in *Setaria italica* was there an associated difference in leaf number and then of only one or two leaves. Low nitrogen has been shown to increase the number of nodes below the first inflorescence of tomato (Wittwer and Teubner, 1957) and to retard progress to flowering in winter rye (Gott *et al.*, 1955), but again these effects were small relative to that found for carnation.

It appears that effects of inorganic nutrition on floral initiation and development are generally more pronounced under conditions otherwise unfavourable to flowering. The present investigation has shown that effects of low nitrogen supply on flower initiation in carnation occur only in photoperiods unfavourable to flowering, namely short days. Similarly Scully *et al.* (1945), working with varieties of soybean which flowered most rapidly in short days, found that nitrogen nutrition only affected flower initiation in long days. Gott *et al.* (1955) showed that nitrogen deficiency retarded progress to flowering in unvernalized and slightly vernalized winter rye but that it was without effect on highly vernalized plants. In no instance, however, has variation in nitrogen supply been found to induce floral initiation under photoperiodic or temperature régimes where this would not otherwise occur (Neidle, 1939; Naylor, 1941; Withrow, 1945; Hinnawy, 1956). Thus where nitrogen nutrition does affect flower initiation it is probably through mechanisms less specific than those controlled by photoperiodic or vernalization treatments.

It is supposed that under the conditions of nitrogen deficiency obtaining in the experiments here, levels of metabolites at the stem apex were reduced to such a level that floral initiation was delayed while the production of leaves could continue but only at a reduced rate. On the basis of this hypothesis it is not obvious why nitrogen deficiency should have an accelerating instead of a retarding effect on floral initiation in some plants. It is not impossible, however, that in these cases growth centres normally in competition with the primary site of flower initiation were in effect eliminated by lack of nitrogen. Thus von Denffer (1940) showed that where nitrogen deficiency hastened flowering in barley and wheat, tillering was suppressed and growth was restricted to the main axis, while in plants receiving a full nutrient treatment the growth of tillers was such that they developed their own inflorescences. It is thus feasible that certain metabolites were more readily available to the stem apices of the nitrogen deficient plants owing to the absence of competing growth centres. In carnation, on the other hand, even in plants receiving the full nutrient treatment, apical dominance was such that there was little development of lateral buds until after floral initiation had occurred at the main stem apex. Hence effects of nitrogen deficiency on the primary site of floral initiation were presumably not complicated by effects on other competing growth centres.

ACKNOWLEDGEMENTS

Part of this work was carried out during the tenure by one of us (J. B.) of a Ministry of Agriculture post-graduate scholarship. Part of the work was supported by a grant from the Agricultural Research Council.

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Life History and Reproduction of *Furcellaria fastigiata* (L.) Lam.

1. The Haploid Plants and the Development of the Carposporophyte

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With two Plates and five Figures in the Text

ABSTRACT

This paper describes the periodicity of the events in the life cycle and the development of the female sexual organs together with the subsequent growth of the carposporophyte within the female plant. The diploid carposporophyte which lives for one year is of the diffuse type and belongs to class C in Drew's (1954) grouping of carposporophytes. The mode of origin of the gonimoblasts and the nature and history of the auxiliary cell are remarkably similar to those of *Halarachnion ligulatum* (Woodw.) Kütz. Chromosome counts of the different thalli and the nature of meiosis in the tetrasporangium (part 2) show that in Britain, and in all probability in the Baltic, the species is a typical dimorphic diplohaplobiont or Drew's (1955) -a-B-A-a- type.

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INTRODUCTION

FURCELLARIA FASTIGIATA (L.) Lam., a common perennial seaweed occurring in the lower intertidal and upper infralittoral zones of shores in the North Atlantic, was placed by Schmitz and Hauptfleisch (1897) in the family Nemastomaceae belonging to the order Cryptonemiales. *Furcellaria*, together with *Halarachnion* and *Neurocaulon*, is held by Kylin (1937) to constitute the family Furcellariaceae which is included in the order Gigartinales. This view is accepted here, following Kylin's classification as exemplified

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[1] The work described in this paper was carried out at the Department of Botany, University College of Wales, Aberystwyth.

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

in the modified key proposed by Papenfuss (1951). The nomenclature of this species has been dealt with by Drew (1958).

The main anatomical features were described by Caspary (1850), Wille (1887), Oltmanns (1922), and Denys (1910). These are summarized by Rosenvinge (1917) and Fritsch (1948). Rosenvinge also elaborated the descriptions of the antheridia given by Thuret (1855) and Buffham (1888), and Grubb (1925) gave a more recent account of these structures, although in none of these investigations was modern cytological technique employed. Rosenvinge (1917) and Kylin (1923) dealt briefly also with the structure of the almost mature carposporophyte. Rosenvinge (1917) alone has made any attempt to relate the development of reproductive structures to a seasonal pattern. Thus no detailed account of the cycle of events in the life history appears to exist, and this is difficult to understand in view of the economic importance of *Furcellaria* (Lund and Bjerre-Petersen, 1952; Austin, 1959*a*).

MATERIAL AND METHODS

Material for study of the life history was collected or received at all seasons of the year and from Kent, Cornwall, Glamorganshire, Pembrokeshire, Anglesey, Scotland, and Denmark. The plants growing at Aberystwyth and on neighbouring shores were examined at each fortnightly spring tide, collections being made daily during the period of meiosis and when receptive trichogynes were developing.

Sections cut on the freezing microtome and squash preparations were found to be the most useful means of investigating the anatomy, particularly for tracing the extensive and diffuse carposporophyte and for observing the minute nuclei. A variety of fixing fluids was used, including Carnoy's fluids, formalin-acetic-alcohol, formalin-alcohol, Karpechenko's, Suneson's, and Flemming's mixtures. Aceto-carmine used after a mordant bath of ferric ammonium sulphate (iron alum) was found to be the most useful stain, although Feulgen's method was also quite successful. The alum-carmine technique proved effective not only as a nuclear stain but also for the demonstration of anatomical details. For the latter purpose the method can be used equally well after almost any fixative, but best results were obtained after formalin-acetic-alcohol or medium strength Flemming's. During the procedure starch, which commonly obscures a great deal, is removed by hydrolysis and the most delicate structures are clearly revealed (Austin, 1959). The thick mucilaginous walls, common in the red algae, are not as heavily stained by this method as by stains such as haematoxylin. The colour does not fade with storage and the method has proved effective with a considerable number of red algae which are generally considered 'difficult material'. The chromosomes of barnacles and of other marine invertebrates have been successfully demonstrated by this method (Austin, Crisp, and Patil, 1958). The material of *Furcellaria* could also be embedded readily in wax and was subsequently stained best in Haidenhain's haematoxylin or by means of Newton's gentian violet iodine method.

SYNOPSIS OF THE LIFE HISTORY

Fig. 1 is a schematic representation of the life-history of *Furcellaria fastigiata* showing the period at which each event takes place, and is based on the observations described in detail below. As in other dimorphic diplohaplobiontic red algae (*sensu* Drew, 1955), there are two nuclear phases and two heteromorphic somatic phases, one of which, the carposporophyte, is parasitic upon the female gametophyte and lives for one year. The gametophytic plants, both male and female, and the tetrasporophyte are perennial. Individual thalli bear reproductive structures when they attain mature length during their fourth to seventh season. This length varies, according to the habitat, from less than 9·0 cm. to over 30 cm. From the uniformity of the annual growth of randomly sampled plants of the three free-living thalli it was deduced that the growth-rates of both gametophytes and of the tetrasporophyte were nearly, but not quite, equal. The tetrasporophytes, which are diploid, are normally the most robust individuals and tend to be more numerous than the gametophytes. However, in their immature state the three types of thallus appear identical and can be distinguished with certainty only by determination of their chromosome numbers.

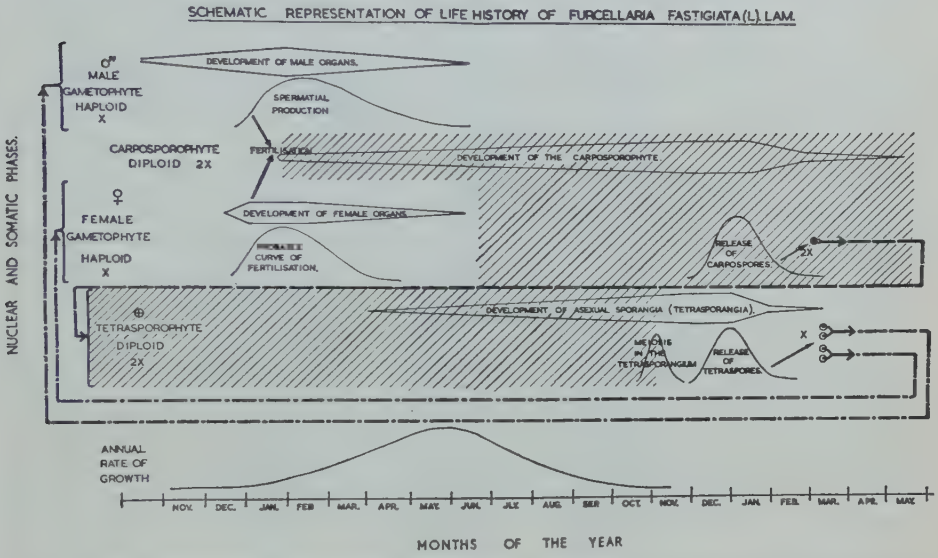
SCHEMATIC REPRESENTATION OF LIFE HISTORY OF FURCELLARIA FASTIGIATA (L.) LAM.
NUCLEAR AND SOMATIC PHASES.
♂ MALE GAMETOPHYTE HAPLOID X
DEVELOPMENT OF MALE ORGANS.
SPERMATIAL PRODUCTION
CARPOSPOROPHYTE DIPLOID 2X
FERTILISATION
DEVELOPMENT OF THE CARPOSPOROPHYTE.
♀ FEMALE GAMETOPHYTE HAPLOID X
DEVELOPMENT OF FEMALE ORGANS.
CURVE OF FERTILISATION.
RELEASE OF CARPOSPORES.
2X
⊕ TETRASPOROPHYTE DIPLOID 2X
DEVELOPMENT OF ASEXUAL SPORANGIA (TETRASPORANGIA).
MEIOSIS IN THE TETRASPORANGIUM
RELEASE OF TETRASPORES.
X
ANNUAL RATE OF GROWTH
NOV. DEC. JAN. FEB. MAR. APR. MAY. JUN. JLY. AUG. SEP. OCT. NOV. DEC. JAN. FEB. MAR. APR. MAY.
MONTHS OF THE YEAR

FIG. 1. The events in the life history of *Furcellaria fastigiata* are here shown in relation to the months of the year. For convenience the winter months are repeated; on the left hand of the figure the development of the haploid plants is shown, and on the right hand the simultaneous events in the carposporophyte and tetrasporophyte. The complete life span from spore germination to death of the plant cannot be included in this scheme, since in such a perennial alga it occupies several years. The diploid part of the cycle is shaded.

Male and female plants are found in equal proportions in natural populations. Tetraspores have been germinated in the laboratory and the haploid nature of the sporelings established, but it has not been possible to grow plants to maturity. Monoecius plants are extremely rare. No previous record seems to exist, but one single specimen bearing spermatangial apices as well as rather stunted swollen ramuli bearing gonimocarps has been found by the present author at Aberystwyth. Sex organs and tetrasporangia have never been seen to occur together on one plant.

Male plants (Fig. 2*a*) are conspicuous in the field between late October and May. The fertile period extends for six months and spermatia are produced continuously from December until April.

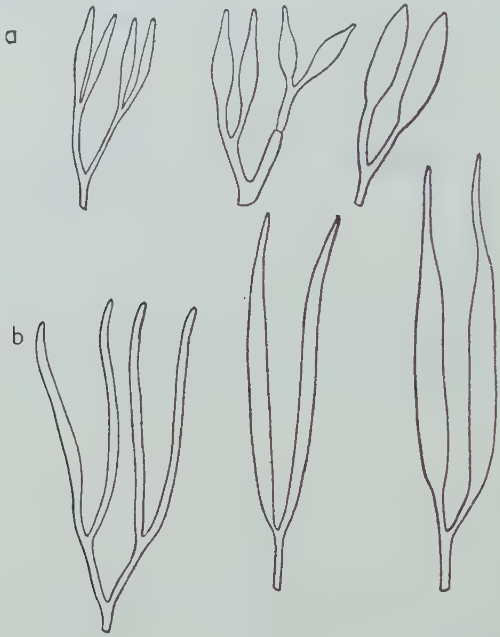

FIG. 2.

(*a*) Male ramuli showing the enlargement which takes place during winter and which occupies between 3 and 6 weeks.

(*b*) Ramuli from female plants showing corresponding elongation and later swelling which extend almost throughout a whole year from early February to December, i.e. the life span of the carposporophyte which lives within these ramuli. ($\times\frac{2}{3}$ approx.)

The reproductive structures of the female plant originate in January, their development ultimately resulting in the production of mature gonimocarps and carpospores almost exactly a year later during December and the following January. Only during the latter half of this period, however, are the plants obviously recognizable as 'fruiting', since only then do the fertile ramuli reach maximum length (3·5–6·0 cm. for Cardigan Bay material) and bear advanced gonimocarps which cause them to swell. Early stages in the development of the female sexual organs can be observed in late December and early January within the apices of mature thalli; these apices become characteristically elongated (Fig. 2*b*), although there are no such conspicuous external changes as seen in the contemporary male plants (Fig. 2*a*). During the first fortnight in January all stages of development from the inception of carpogonial branches to the production of ripe elongated trichogynes have been found. By the end of the month the first gonimoblasts are seen to be developing rapidly; in mid March secondary gonimoblasts appear and the generative auxiliary cells are conspicuous (Pl. 2. 3). On account of the mode of origin of the female organs behind the apical meristem younger stages are to be found nearest the apex.

In material from all localities examined the above developmental sequence occurs at much the same time of year, although plants from the northerly localities and from very exposed places appear to be somewhat slower in development.

According to Rosenvinge (1917) the Danish plants form carposporophytes seven months later than the British ones. However, material collected by the present author at a number of localities in Denmark during 1955 exhibited stages as advanced as those collected on British shores at the same time of year. Rosenvinge's records for antheridial material are comparable with my observations and also those of Grubb (1925) for British plants. This, however, results, in the Danish material, in a gap of three months between the last observed spermatia (in May) and Rosenvinge's first records of carpogonia (in August). It seems likely therefore that the first formed carposporophytes were overlooked by him, since it is very easy to miss the earliest signs of these structures. If this were so then the Danish plants have an annual cycle essentially like that of the British plants.

DEVELOPMENT OF THE SEXUAL REPRODUCTIVE ORGANS OF THE HAPLOID PLANTS

The internal organization of the thallus of *Furcellaria* has been dealt with by Oltmanns (1922) and some further remarks have been made by Austin (1959*a*). The structure is of the fountain type with a central medulla of axial filaments which give rise to closely packed laterals. These constitute the cortex and assimilatory layers, the whole forming a firm cylindrical thallus branching by dichotomy.

Male gametophyte. Although the present investigation confirms the description of the development of spermatangia given by Grubb (1925) as the more usual one, it also suggests that this may sometimes take place in a different manner. Instead of the early formation of a wall between the young spermatangium and the spermatangial mother-cell, the latter appears to bud off the spermatangium by progressive narrowing of a transverse constriction half-way along the vertical axis. Before this takes place the nucleus of the mother-cell divides. The outer spermatangium so formed enlarges, assumes a conico-ovoid shape, and the contents later round up and escape as a single spermatium, the inner lower cell enlarging and sometimes repeating the process.

It was found difficult to observe nuclear division in the spermatangial cells, although attempts were made by fixing material at all hours of the day and night. Prefixation treatment of perfectly fresh material for from 5 to 25 minutes in saturated aqueous dichlorobenzene was found helpful in arresting divisions at late prophase and at metaphase, enabling a larger number of these stages to be observed. The nuclei of the spermatangia and spermatia are barely 3μ–5μ in greatest diameter and spread out when squashed only with difficulty. The chromosomes are highly contracted and are extremely small in size; accurate counts are therefore best obtained from the larger dividing nuclei in the growing apices in March and April. The number of chromosomes was found to be 34 and, since twice this number was counted in the tetrasporangial plant, the male gametophyte, as would be expected, is evidently haploid.

The structure of the superficial layers of the thallus in *Furcellaria*, as seen when stained with haematoxylin and particularly by alum-carmine, strikingly resembles that of *Polyides caprinus* (Gunn) Papenf. (Rao, 1956, p. 214) and also that of the brown alga *Hormosira banksii* (Turn) Decaisne (Osborne, 1944). At the thallus surface the tips of the filaments are closely packed forming a continuous layer; covering this is an additional outer investment of a different constitution which absorbs alum-carmine strongly. This 'cuticle' can be seen to be a continuous layer which, owing to differences in thickness corresponding to the rounded ends of the cells beneath, exhibits a polygonal or honeycombed sculpturing on its inner surface (Pl. 1. 1).

Each spermatium is released through a very definite pore, the rim of which is slightly raised and absorbs carmine strongly. The pores are arranged in groups of two, three, or four, but most commonly of three, indicating a similar arrangement of spermatangia on their mother cells; this is confirmed when seen in section or in squash preparations.

Female gametophyte. Development of reproductive structures in the female plant begins in January and dividing nuclei are easily obtained during February, March, and April from the actively growing apical region. The number of chromosomes was found to be 34—the haploid number—and therefore identical with that found in the male gametophyte.

The supporting cells which bear the fertile accessory lateral or carpogonial branches are cells situated almost at the base of a primary lateral and resemble neighbouring cortical cells. Carpogonial branch initials arise laterally and subapically from these cells. Two commonly arise at first, one on each side of the distal half of the cell followed by a third, a fourth, and even a fifth and sixth in some cases. Growth of the carpogonial branch is apical and rapid, the lower cells being cut off from the base of the elongate pyramidal terminal cell (Fig. 3*a*; Pl. 1. 2); this from an early stage has a small ovoid protuberance at its apex, which later becomes the trichogyne (cf. *Polyides caprinus*—Rao, 1956). When two or three cells have been formed the terminal cell divides horizontally into an upper carpogonium and a smaller hypogynous cell (Fig. 3*a*; Pl. 1. 2); the carpogonial branch is therefore generally 3- to 5-celled.

Carpogonia are produced over a considerable period and young carpogonial

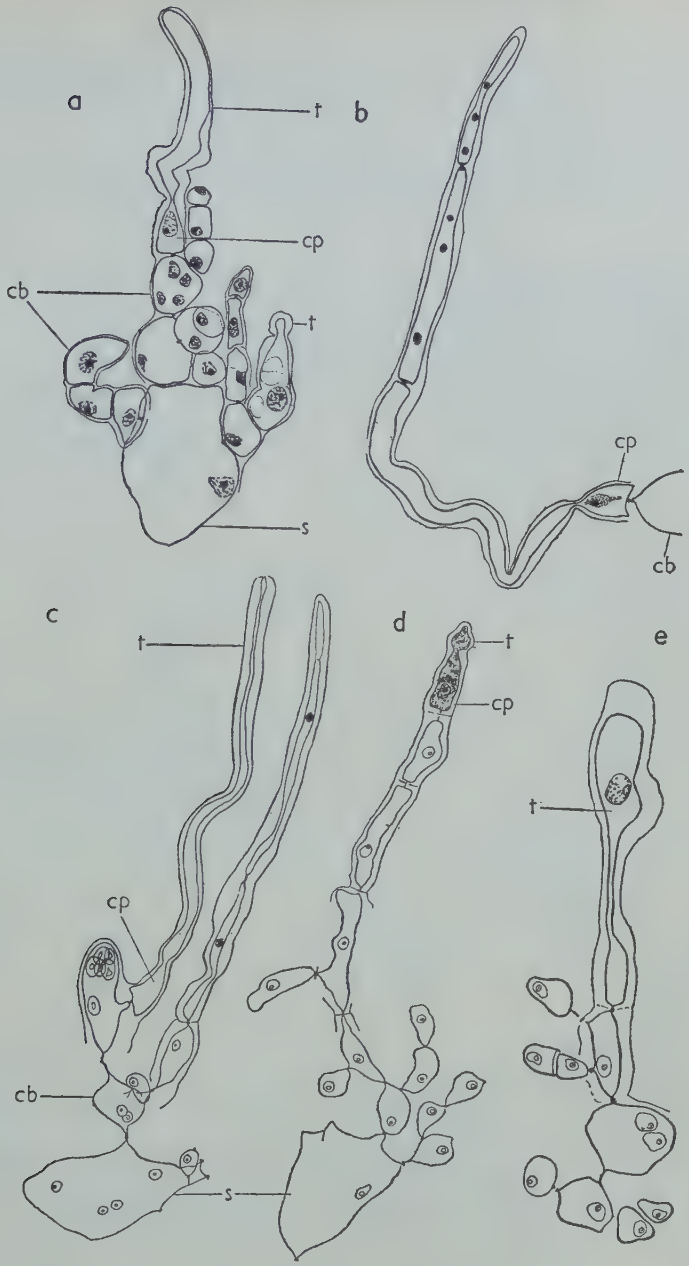

FIG. 3. (All ×475 approx.)

(*a*) Three carpogonial branches (*cb*) arising from a single supporting cell (*s*). One carpogonium (*cp*) bears an almost mature trichogyne (*t*), whilst another smaller one is still in an early stage of development but possesses a rudimentary trichogyne.

(*b*) An abnormal trichogyne represented by a row of ill-developed cells with indistinct nuclei.

(*c*) A normal carpogonium and trichogyne are seen to the left of an abnormal structure, where the female organs are represented by a row of three cells.

(*d*) A small ill-developed carpogonium and trichogyne terminating an accessory branch which resembles a secondary hyphal lateral rather than a carpogonial branch.

(*e*) An enucleate carpogonium with an irregular nucleate but unreceptive trichogyne, *t*.

branches can be seen when secondary gonimoblasts are forming. However, it seems that the carposporophytes are all of more or less the same age and it is inferred from this and other observations that fertilization only takes place over a short period of time (Fig. 1).

The carpogonium itself increases slightly in size but the small protuberance at its apex elongates, eventually breaking the surface of the thallus as a mature, enucleate trichogyne (Fig. 4*a*, *b*, *c*). The carpogonium itself remains bullet-shaped and when very young its protoplast consists of dense but homogeneous cytoplasm containing a single nucleus.

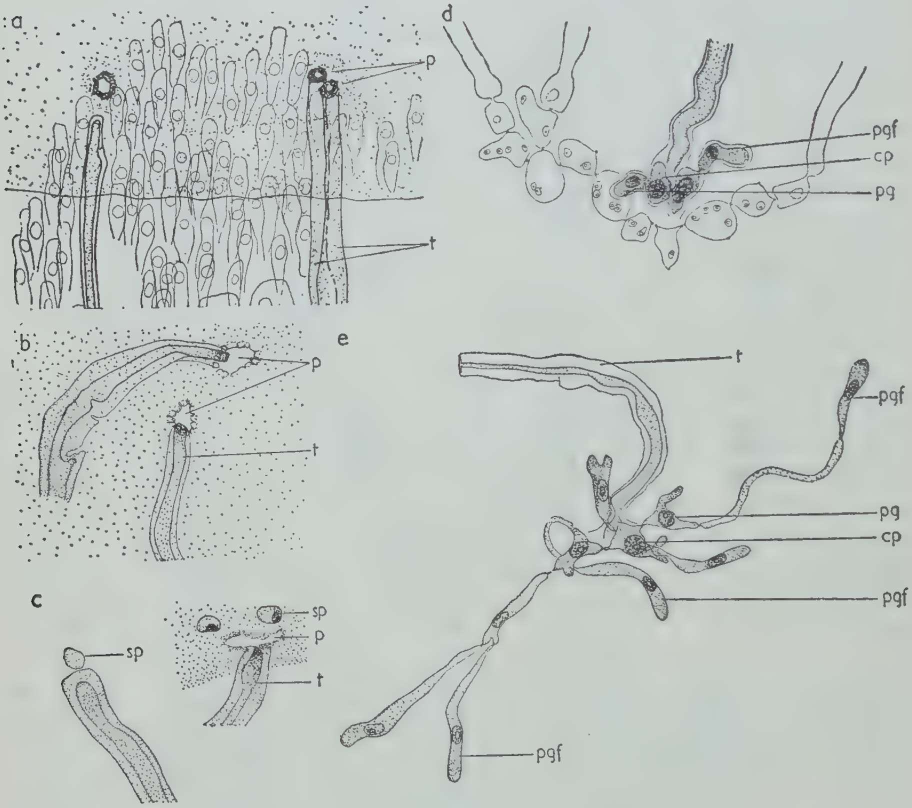

FIG. 4. (All ×325 approx.)

(*a*) (*b*) (*c*) Drawings of the receptive tips of mature elongate trichogynes (*t*) at the surface of the thallus. The pores (*p*) in the 'cuticle' or outer investment can be seen, together with spermatia (*sp*) adhering to the latter.

(*d*) (*e*) Early stages in post-fertilization development. (*d*) The first divisions of the fertilized carpogonium (*cp*) into very dense cells, the primary gonimoblasts (*pg*), which elongate into filaments (*pgf*). (*e*) A slightly later stage, when the carpogonium has given rise to those cells with large diploid nuclei, each of which produces a primary gonimoblast filament (*pgf*).

A carpogonial branch may frequently give rise to lateral branches, generally from the two or three lower cells, but occasionally from the hypogynous cell

(Fig. 3*a*, *b*, *e*; Pl. 1. 2). These laterals are 2- to 3-celled and may or may not terminate in a carpogonium (Pl. 1. 2).

Each cell of the thallus has one nucleus, but the number of nuclei in the cells of the carpogonial branch, all originally uni-nucleate, later becomes variable; in most cases the lower (proximal) cells have two nuclei but they may have one, three, or four. When the trichogyne begins to elongate the hypogynous cell enlarges considerably and its nucleus divides to give two, four, and then eight nuclei, although the odd numbers seven and five are frequently seen (Fig. 3*a*, *b*; Pl. 1. 2). This multi-nuclear condition might be indicative of arrested branching (cf. Kylin, 1930).

Certain deviations from this normal course of development have been observed.

(*a*) The distal half of a trichogyne may be represented by a short row of 2–4 cells (Fig. 3*b*).

(*b*) Both the carpogonium and trichogyne may be replaced in a similar manner by 2–4 elongated cells (Fig. 3*c*).

(*c*) A carpogonium with a very short trichogyne has been seen terminating an ill-formed carpogonial branch (Fig. 3*d*).

(*d*) An enucleate carpogonium may have a swollen nucleate trichogyne (Fig. 3*e*).

(*e*) The terminal cell of a carpogonial branch may be elongate and of irregular shape, and hardly recognizable as a carpogonium.

Thus the carpogonial branch may be replaced by a vegetative lateral and appears therefore to be homologous with an accessory or secondary lateral branch arising from an undifferentiated cell (the supporting cell) near the base of a primary lateral of limited growth. The carpogonium and trichogyne probably originate from the single apical cell of such a lateral, since both may be replaced by a single vegetative cell and always have a single nucleus between them. However, the trichogyne may be replaced by more than one cell, and it is possible that the carpogonium and trichogyne represent the arrested development of more than one cell.

A single supporting cell typically bears three to four carpogonial branches which exhibit some synchronization in their growth, particularly in the rapid elongation of the trichogynes. The trichogyne, which is sometimes swollen and irregular in shape, elongates to reach the firm superficial investment of the thallus, where it causes an irregular pore to be formed (Fig. 4*a*, *b*, *c*).

It is possible that the tip of the trichogyne may project beyond the surface of the thallus, but, since these structures have been best seen in rather thick sections of frozen material, a certain degree of displacement might have occurred. Penetration of the thallus surface is, however, well marked (Fig. 4*a*, *b*). In one or two instances spermatia have been found on the thallus surface near the tips of mature trichogynes (Fig. 4*c*). However, despite the examination of a very large amount of material, the actual process of syngamy was not observed, although several instances of an ill-defined nucleus within the trichogyne have been seen.

Development of the Carposporophyte

The largest number of mature elongated trichogynes was met with in the period between late January and mid-February. During the latter half of this period the prolific growth of primary gonimoblast filaments was very conspicuous. This marks the beginning of the carposporophyte phase which remains parasitic upon the female gametophyte until carpospores are formed and shed one year later. The diploid nature of the carposporophyte has been verified by chromosome counts made on the dividing nuclei in gonimoblast filaments (Figs. 4*d*, *e*), as well as at the division of the nucleus—a derivative of that of the carpogonium—which enters the generative auxiliary cell (Pl. 2. 1; Fig. 5*a*). The female gametophyte which nourishes the carposporophyte is haploid (p. 262) as is the male plant (p. 262). Since the tetrasporophyte has been shown to be diploid (part 2) and meiosis in the tetrasporangium quite normal (part 2 and Austin, 1955), the life cycle would appear quite normal and there

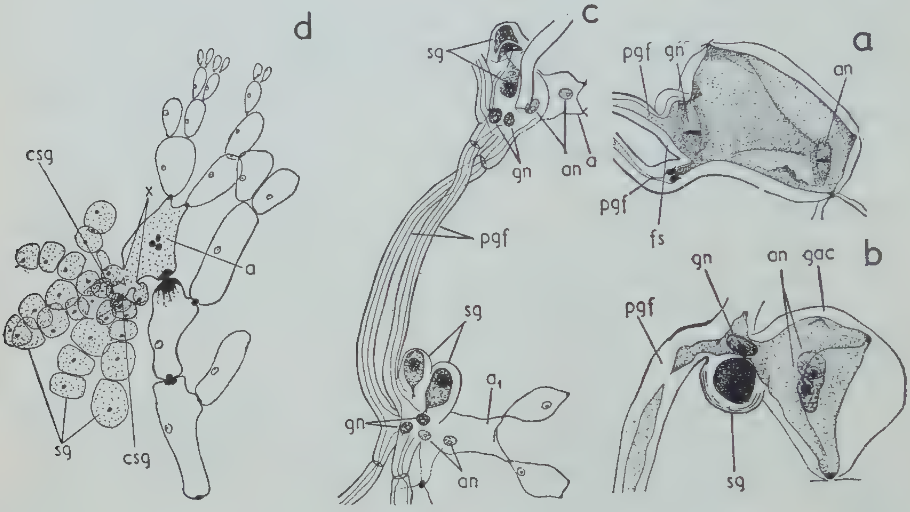

FIG. 5.

(*a*) A primary gonimoblast filament (*pgf*) has fused with a generative auxiliary cell (*gac*) and a 'fusion swelling' (*fs*) has been produced at the point of contact. The haploid nucleus of the auxiliary cell (*an*) and the diploid nucleus of the gonimoblast (*gn*) are shown in simultaneous mitosis. (×450.)

(*b*) The first secondary gonimoblast (*sg*) has been cut off and contains one of the products of the division of the diploid primary gonimoblast nucleus; the other daughter diploid nucleus (*gn*) remains in the auxiliary cell, together with the two daughter nuclei (*an*) produced by the division of the haploid auxiliary cell nucleus. (×450)

(*c*) Duplication of events shown in Fig. 5*b* by the simultaneous fusions between two primary gonimoblast filaments (*pgf*) and an auxiliary cell (*a*). Since the two gonimoblasts proceed to fuse with another auxiliary cell (a_1) the whole process is repeated. (×175)

(*d*) A cluster of secondary gonimoblasts (*sg*) radiating from the group of small central first formed secondary gonimoblast cells (*csg*). The latter can be seen to have wide connexions (*x*) with the auxiliary cell (*a*). The cells of the lateral, to which the auxiliary cell belongs, can be seen to have large nuclei and huge pit connexions with each other. (×150) is no reason to believe that fertilization does not take place (see p. 270 'Discussion').

The primary gonimoblasts arise from the carpogonium or from the cell or cells produced by its division subsequent to fertilization (cf. *Halarachnion ligulatum* Kylin, 1928). Figs. 4*d* and *e* illustrate stages in the production of the primary gonimoblasts.

The carpogonium, after fertilization, becomes separated from the trichogyne at the constriction between them and enlarges. Its large diploid (zygotic) nucleus divides repeatedly, each daughter nucleus passing into a protuberance which is cut off at once as a primary gonimoblast (Fig. 4*d*). These may develop forthwith into elongate filaments or produce one or more cells, similar to the now polyhedral fertilized carpogonium, all of which give rise to gonimoblast filaments in varying number (Pl. 1. 3; Figs. 4*d*, *e*). These primary gonimoblast filaments then exhibit a form of lateral branching and increase rapidly in number. Their ramifications are so extensive, obscuring much of the original carpogonial branch, that it is sometimes difficult to trace each cell back to its origin. Adjacent carpogonial branches appear to cease development subsequent to the appearance of gonimoblasts and changes, which might be degenerate, occur.

The primary gonimoblast filaments, which vary from 8μ to 15μ in width, have club-shaped apices with dense granular cytoplasm and each cell has a large diploid nucleus with a prominent nucleolus (frequently there are two nucleoli) and with deeply staining heterochromatic regions. These filaments ramify through the middle and inner cortex and even through the medulla.

At this time another series of cells becomes progressively more conspicuous. These, like the supporting cells, lie near the bases of primary laterals and are those with which the primary gonimoblasts fuse and from which the secondary gonimoblasts arise; they must therefore be called generative auxiliary cells (*sensu* Drew, 1954). Each originates by metamorphosis of an ordinary inner cortical cell and comes to possess distinctive characteristics before fusion with the primary gonimoblast. The nucleus is very large and commonly lies in the outer or distal half of the cell. It appears to be in a state resembling that of early mitotic prophase (Pls. 1. 4, 5; 2. 3). The cell contents become granular and very densely staining, tending to accumulate near the conspicuous pit-connexions with the other cells of the lateral, which also have granular contents and large nuclei (Pl. 2. 3). There is normally no chromatophore, although remnants may be present as small disc-shaped bodies which are irregularly distributed within the cytoplasm.

A primary gonimoblast filament commonly approaches an auxiliary cell from below (Pl. 2. 3), and touches the proximal part of the cell which tends to protrude (Pl. 1. 5). The nucleus of the gonimoblast divides, one daughter nucleus passing to the apex of the filament, which, continuing its rapid growth, bends away from the auxiliary cell (Pl. 1. 5, 6). A septum forms between the growing tip of the gonimoblast cell and point of fusion with the auxiliary cell. Fusion and open contact is established between the penultimate cell of the gonimoblast filament and the auxiliary cell. This common region, or 'fusion swelling', has a conspicuous wall or cell sheath (Fig. 5*a*, *b*; Pls. 1. 6; 2. 3).

The two nuclei involved, one the haploid gametophytic nucleus in the upper (distal) half of the cell, and the other the diploid gonimoblast nucleus within the fusion swelling or within the lower half of the auxiliary cell, enter prophase (Pls. 1. 6; 2. 1). However, as they do so and before they divide, a definite protuberance with extremely dense contents and conspicuous cell wall is produced on the fusion swelling (Pl. 1. 6). This protuberance occurs on the lower portion of the generative auxiliary cell but is outside (distal to) the fusion swelling. The two nuclei divide (Pl. 2. 1), exhibiting parallel spindles (Fig. 5*a*). One of the nuclei produced by the division of the diploid nucleus from the gonimoblast passes at once into the protuberance and is cut off as the first cell of the secondary gonimoblast (Fig. 5*b*; Pl. 2. 2).

Rarely, both the nuclei produced by the division of the diploid nucleus pass into separate protuberances and cause twin secondary gonimoblast initials to be cut off. More commonly the remaining (diploid) nucleus moves slowly toward the centre of the auxiliary cell, whilst the two nuclei produced by the division of the original (haploid) nucleus of the auxiliary cell also migrate to a more or less central position. These three nuclei tend to enlarge, stain faintly, appear diffuse, and exhibit nucleoli and heterochromatic granules characteristic of a resting state. They take no further part in development (Fig. 5*b*, *c*, *d*; Pl. 2. 2, 5). The sequence of events described here is remarkably parallel to the pattern seen in *Halarachnion ligulatum* (Woodw.) Kütz (Kylin, 1928).

The following deviations from this pattern of events can occur:

(i) Occasionally the four nuclei formed by the synchronous division (two haploid, two diploid) pass well into the auxiliary cell before further development.

(ii) Two gonimoblast filaments may contact a single generative auxiliary cell; the events described above are duplicated, two secondary gonimoblast initials are cut off, the remaining diploid nuclei passing into the auxiliary cell, where four nuclei (two diploid and two haploid) are then found (Fig. 5*c*).

(iii) One example of three filaments contacting a single auxiliary cell has been seen, but it appears that three secondary gonimoblasts were cut off without the division of the diploid nuclei or of the haploid nucleus, since only one (haploid) remaining nucleus could be seen in the auxiliary cell.

(iv) In one instance early stages of the growth of a secondary gonimoblast have been seen to occur on a primary gonimoblast close to a dichotomy or junction of the latter, with no contact or fusion with an auxiliary cell or any cell of the gametophyte.

The first secondary gonimoblast cell is dome-shaped with a large nucleus and very dense contents (Fig. 5*b*). It enlarges somewhat, becomes ovoid and after division of its nucleus a second cell is cut off distally (Pl. 2. 2). This cell in turn cuts off a number of cells around its distal half by means of oblique walls. These cells remain small and their contents dense, and constitute a more or less well-defined group radiating from the first-formed secondary gonimoblast, which comes to resemble a 'beak' or outgrowth of the auxiliary cell due to the massive enlargement of the pit connexions between the two (Pl. 2. 4; Fig. 5*d*). Occasionally the second and third formed cells of the secondary gonimoblast also form conspicuous connexions with the auxiliary cell (Fig. 5*d*).

From an early stage in the development of the carposporophyte, the cells of the primary lateral, of which the auxiliary cell is a member, become differentiated from those of neighbouring laterals by the possession of dense contents, rather large nuclei, and wide pit-connexions with each other (Pl. 2. 3). As the secondary gonimoblasts develop, these features become more marked and can be traced back along the length of the lateral to its insertion in the upper (distal) end of an axial filament cell (Fig. 5*d*; Pl. 2. 6). The latter figure also illustrates the accumulation of substances which have a protein reaction at the upper (distal) parts of the axial filament cell.

Concurrently with the development of the first secondary gonimoblasts, the auxiliary cell itself normally enlarges and its contents remain deeply staining although without chromatophores. The number of nuclei in this cell may remain at 3 (Fig. 5*d*; Pl. 2. 2) or these may divide rather haphazardly to four, five, six, or even seven small densely staining nuclei with a pycnotic appearance.

The cell walls of the outer secondary gonimoblasts are very conspicuous and in the early stages form a well-marked envelope delimiting the secondary gonimoblast cluster or gonimolobe. However, at a later date hyphal filaments, which have their origin in cells of the inner cortex and medulla, grow towards and around the developing gonimoblasts. When the latter are almost mature and have increased greatly in number, the surrounding hyphal filaments are compressed into narrow, rather dense bands which then appear to form an ill-defined pericarp or delimiting and protective layer around the gonimolobe (Drew, 1954) constituting a gonimocarp or micro-gonimocarp. The latter now consists of a mass of radiating compact filaments, almost all the cells of which become carposporangia, but, despite continued growth and expansion until the time of spore discharge, the actual formation of new gonimoblast cells ceases a month or more earlier.

The carpospores are produced singly in the carposporangia and are quite large, though compressed by mutual pressure from neighbours within the thallus to a somewhat polyhedral shape. Approximately 200 spores are produced in a gonimolobe (= cystocarp) of average dimensions. There are perhaps 150 such gonimolobes in a fertile ramulus, and 30 such ramuli per female thallus, giving 900,000 or almost a million spores per plant. This number is inevitably an approximate one, for considerable differences occur in the dimensions of plants from different habitats (Austin unpub.) with corresponding differences in size of fertile ramuli and cystocarps. Whether spore size is also affected has not been investigated. Almost all the spores formed are released during the period between 24–28 December and 14–28 January.

Directly above (distal to) the gonimolobe, which may have a diameter of one-third of that of the now swollen fertile ramulus, the contents of certain cells of the cortex and assimilatory layer become granular, more deeply stained, and tend to shrink. These cells belong to the primary lateral in which the auxiliary cell (of the particular gonimolobe) occurs. Their cell walls swell and later separate, the eventual disorganization of the tract of cells forming a more or less well-defined duct from the gonimolobe to the surface of the thallus, where it opens by an ill-defined pore. Thalli with fertile ramuli discharging ripe carpospores were immersed in sea water and examined directly after removal from the sea. The spores were exuded through the above described duct and were held together in little heaps on the surface of the thallus by the copious mucilage exuded with them. They were ovoid or somewhat polyhedral on emergence through the pore, but rounded up rapidly to assume a diameter of between 35μ–50μ. Commonly about half a dozen spores were squeezed one after another out of the duct, and a short interval occurred before another such batch appeared. The mechanism of spore emission probably depends on increase in internal pressure in the microgonimocarp due to the secretion of copious mucilage which imbibes water strongly.

The carpospore is spherical and has a very thin wall. The chromatophores consist of small lenticular segments interconnected to a greater or lesser extent and interspersed with abundant grains of Floridean starch. The nucleus is more or less centrally placed and is in a resting condition, with a conspicuous nucleolus, and the cytoplasm of the cell is densely granular.

Attempts have been made to germinate and grow the carpospores and tetraspores under a variety of conditions both in the laboratory and in the field. Since these experiments were identical for both types of spore, and the results obtained comparable, the details are given in a later paper dealing with the tetrasporophyte (Austin, part 2).

Discussion

Numerous carpogonial branches are borne on a single supporting cell (cf. *Halarachnion ligulatum* (Kylin, 1928)) resulting in a considerable number of carpogonia and trichogynes in a fertile ramulus. A large proportion of these trichogynes reach the thallus surface and become receptive at a period coinciding exactly with peak production of spermatia from male plants. However, few spermatia have been seen attached to ripe trichogynes and the immediate pre-fertilization behaviour of the gametic nuclei is not known. It is likely that only a few of the mature carpogonia within a fertile ramulus are fertilized and these constitute the very active centres from which zygotic nuclei are carried, by means of the numerous and extremely vigorous primary gonimoblasts, to auxiliary cells throughout the ramulus. The nuclei of the primary gonimoblasts have 68 chromosomes and are therefore presumably diploid, for both male and female gametophytic thalli have been seen to possess 34 chromosomes. Somatic divisions in the actively growing apices of the tetrasporophyte also revealed the diploid number of chromosomes, whilst the sporelings produced by the germination of the tetraspores were haploid. A normal meiosis occurs in the tetrasporangium prior to the formation of the four zonate spores (Pt. 2).

Thus it seems certain that *F. fastigiata* possesses a life history of the typical diplobiontic type. The post fertilization changes in this species resemble most closely those of its close ally, *Halarachnion ligulatum*, where the carpogonium, after fertilization, immediately enlarges and undergoes divisions and the products give rise to numerous primary gonimoblasts (Kylin, 1928). A similar sequence of events is encountered in *Agardhiella tenera* (Solieriaceae; Kylin, 1928) and a parallel within the Cryptonemiales is seen in *Grateloupia filicina* (Wulf) Ag. (Kylin, 1930).

Rosenvinge's evidence for parthenogenesis is difficult to entertain, since it seems to be based upon the origin, from an immature carpogonium, of a primary gonimoblast. The latter does not, in fact, arise from the carpogonium in the manner he depicts (1917, p. 168, Fig. 85). Moreover, the vital point of origin of the gonimoblast is, in his figure, obscured by an overlying cortical cell.

The formation and behaviour of the auxiliary cell and its nucleus again exhibit close parallelism with *Halarachnion* and other Gigartinales without procarps, such as *Platoma bairdii* (Farl.) Kuck, where the auxiliary cell is of the generative type. However, in *Furcellaria*, as in *Bertholdia neapolitana* (*Calosiphonia neapolitana*) and *Nemastoma*, the secondary gonimoblast rudiment often appears before the diploid nucleus enters the fusion swelling. Therefore the behaviour of the auxiliary cell in *Furcellaria* is not as sharply distinguished from that in *Polyides* as one would at first sight suspect (Rao, Thesis, 1954). On one occasion in the present investigation the secondary gonimoblast was seen to arise from a primary gonimoblast which had made no fusion with a cell of the gametophyte.

Normally the secondary gonimoblast initial is cut off proximally (internally) upon the swelling formed at the point of fusion between auxiliary cell and primary gonimoblast. The auxiliary cell at this point becomes elongated into a process or beak with which the small first-formed secondary gonimoblasts make conspicuous, often large and open, connexions. The presence of this central group of cells which remain sterile, together with the tendency toward partial fusion between them and the auxiliary cell, appears to constitute a rudimentary 'placental' or nutritive tissue (cf. *Agardhiella tenera* and other Soliercaceae). Furthermore, as the gonimolobe develops, the cells of the primary lateral, in which the auxiliary cell is situated, become markedly differentiated by virtue of their dense contents and huge pit-connexions with each other and with the axial filaments from which the lateral springs. These axial filaments exhibit dense accumulation of substances at their upper (distal) ends (Pl. 2. 6). This might indicate that some translocation of nutritive substances (with some build-up of those below the pit-connexions) takes place through the large well-developed axial filaments and their laterals, particularly towards the developing gonimolobes. These developments may constitute the rudiments of a primary nutritive tissue (cf. *Agardhiella tenera* and others) and may be of considerable importance, for, as in related genera, nutrients reach the secondary gonimoblasts mainly by way of the auxiliary cell.

The present investigation has shown that the earliest stages in the origin of the carposporophyte, the type and history of the auxiliary cell and gonimolobes, and the origin of the tetrasporangia (Austin, Pt. 2) are all different from the corresponding events in *Polyides* (Rao, 1956). Whilst there are certain resemblances between *Furcellaria fastigiata* and *Polyides caprinus*, notably their external appearances and the possession of diffuse carposporophytes, the differences seem to be more important. In fact, in its structure and internal organization, *Furcellaria* again closely resembles *Halarachnion ligulatum* and also *Schizymenia dubyi* (Chauv.) J. Ag., despite the considerable differences in external appearance between the three species; in addition, *Furcellaria*, like *Schizymenia*, has a well-defined pore above each mass of carpospores. There would seem therefore to be no reason to make any change in the present classification of *Furcellaria fastigiata* proposed by Kylin (1937, 1956).

ACKNOWLEDGEMENTS

This investigation was carried out at the Botany Department, Aberystwyth. I am indebted to Professor L. Newton, who suggested the investigation, and to Dr. M. T. Martin and Dr. D. J. Crisp for many helpful criticisms in the preparation of the manuscript for publication. I wish also to thank Dr. H. Blackler, Mr. H. T. Powell, Miss D. Ballantine, Dr. W. E. Jones, and Dr. A. J. Lacey for sending me material, often during inclement weather conditions.

SUMMARY

1. The sex organs develop during late winter within the gametophytes which are diecious and perennial. Both male and female plants have the haploid number of chromosomes, which is 34.

2. Fertilization appears to occur in late January and early February and only a few carpogonia in a whole fertile ramulus are fertilized.

3. Numerous primary gonimoblasts are produced by the germination of the fertilized carpogonium and multiply very rapidly by dichotomy, ramifying throughout the fertile ramulus.

4. The primary gonimoblasts fuse at points remote from their origin with generative auxiliary cells, which are intercalary cells of ordinary vegetative laterals; the latter constitute a primary nutritive tissue. Simultaneous division of the haploid (auxiliary cell) and diploid (gonimoblast) nuclei occurs, and one of the latter nuclei is cut off in the secondary gonimoblast. The latter

forms small central nutritive cells, which may partially fuse with the auxiliary cell, and which give rise to filaments terminating in carposporangia; the whole enlarges and matures to a gonimocarp, which liberates ripe carpospores in December and early January, exactly one year after the appearance of the female organs. A single plant may produce almost a million carpospores.

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EXPLANATION OF PLATES 1 AND 2

PLATE 1

FIG. 1. A portion of the 'cuticle' or outer investment of the thallus showing honeycombed sculpturing on lower surface. (×700)

FIG. 2. Four carpogonial branches (*cb*) with trichogynes at various stages of development (t_1, t_2) borne on a single supporting cell (*s*). (×500)

FIG. 3. Primary gonimoblast filaments (*pg*) springing from a fertilized carpogonium (*cp*) which has enlarged and disjoined from its trichogyne (*t*). (×650)

FIG. 4. Generative auxiliary cell showing greatly enlarged nucleus in a state resembling early prophase, dense granular contents, and lack of chromatophore. (×750).

FIG. 5. Primary gonimoblast filament (*pg*) about to fuse with receptive protuberance on generative auxiliary cell (*ga*). (×600)

FIG. 6. Shortly after fusion the nuclei of the gonimoblast (*gn*) and auxiliary cell (*an*) enlarge and enter prophase. The secondary gonimoblast initial is already being formed and is very conspicuous (*sg*). (×600)

PLATE 2

FIG. 1. Simultaneous divisions of the haploid (gonimoblast) nucleus (*gn*) and diploid (auxiliary cell) nucleus (*an*) in the auxiliary cell. Both nuclei are in late mitotic prophase. The secondary gonimoblast initial (*sg*) is now very pronounced. (×700)

FIG. 2. The latter initial has been cut off and is now two-celled. Three residual nuclei, two haploid (hn_1, hn_2) and one diploid (*dn*), can be seen in the auxiliary cell. (×700)

FIG. 3. Part of a thick longitudinal section of a fertile ramulus in later February, showing primary gonimoblasts (*pg*) which have fused with auxiliary cells (*a*) with the production of the very first secondary gonimoblasts (*csg*). (×85)

FIG. 4. The magnified centre of a group of young secondary gonimoblast filaments (*sgf*) to show the central group of small sterile first-formed cells (*csg*), together with their wide connexions (*x*) with the auxiliary cells (*a*). (×300)

FIG. 5. A group of secondary gonimoblasts (*sg*) (less highly magnified than 4) to show the relative positions of the auxiliary cell (*a*), primary gonimoblast (*pg*), and cells of the lateral to which the auxiliary cell belongs. (×220).

FIG. 6. A thick transverse section of a fertile ramulus in April to illustrate the young secondary clusters (*sg*) and the swollen cells and huge pit connexions of the lateral (*l*) joining the auxiliary cell (*a*) to the axial filament (*af*) in the medulla of the thallus. (×90)

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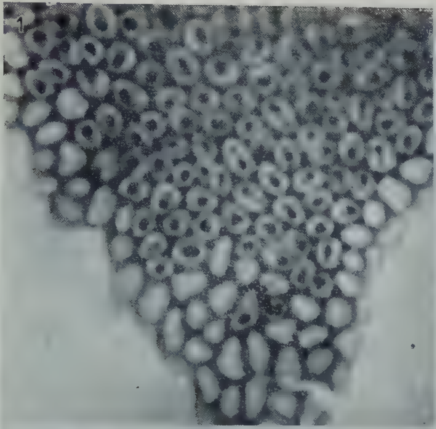

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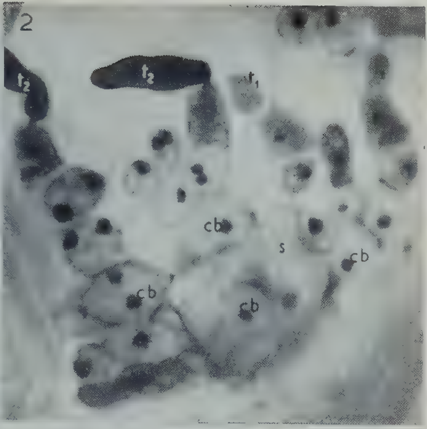

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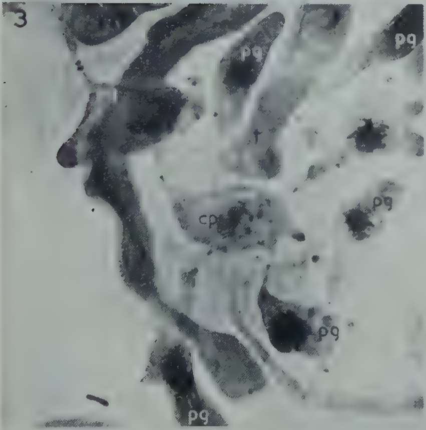

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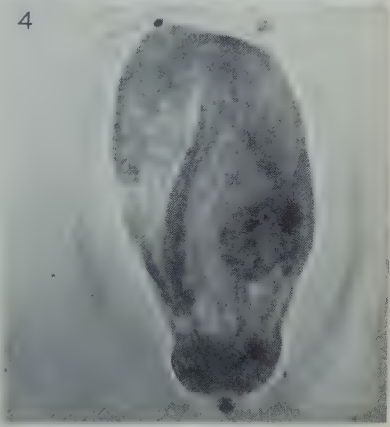

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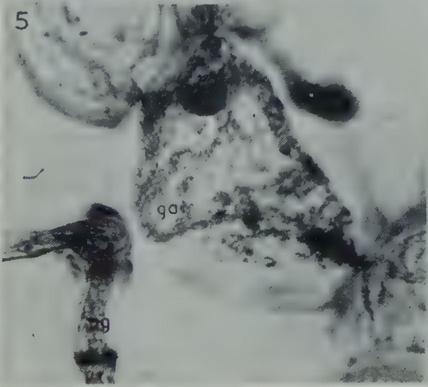

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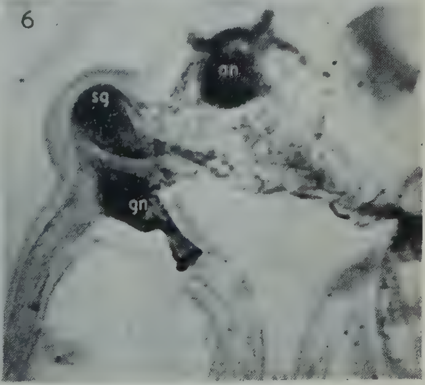

A. P. AUSTIN

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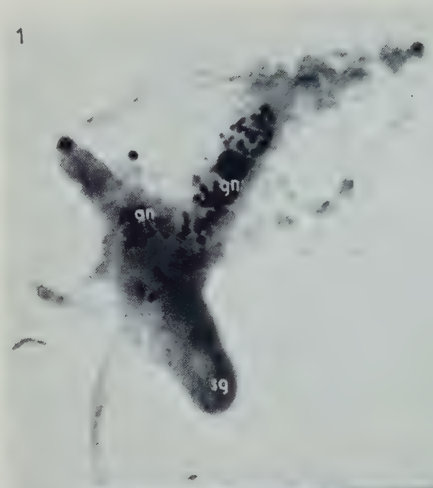

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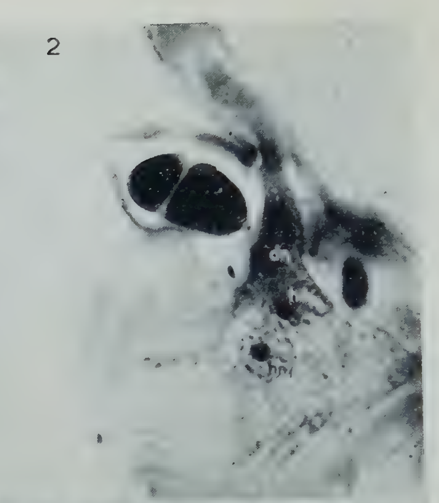

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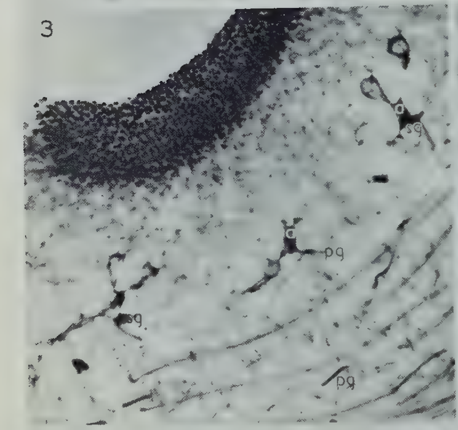

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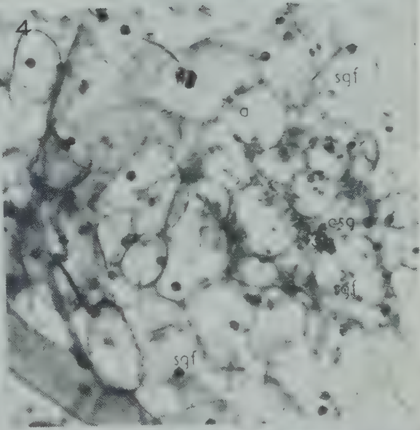

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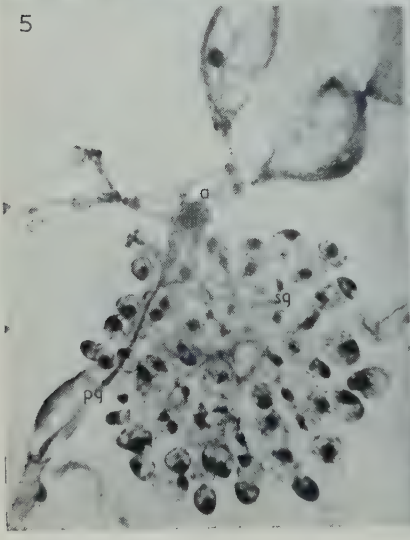

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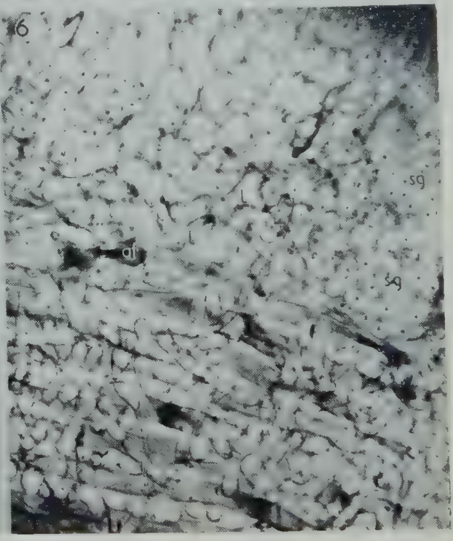

A. P. AUSTIN

Rhizomorph Behaviour in Armillaria mellea (Fr.) Quél.

III. Saprophytic Colonization of Woody Substrates in Soil

BY
S. D. GARRETT
(*Botany School, University of Cambridge*)

With one Figure in the text

ABSTRACT

Rhizomorphs put out by *Armillaria mellea* from small woody inocula in glass tubes of moist soil were tested for their ability to colonize segments of willow shoots, previously killed by autoclaving and then buried for various periods in soil before inoculation with *A. mellea*. The degree of saprophytic colonization of these substrate segments by *A. mellea* was assessed by reburying them in fresh tubes of moist soil, and recording weekly growth increments of rhizomorphs put out from them over a period of 5 weeks. A period of previous burial in soil up to 3 weeks was found not to diminish availability of the substrate segments to *A. mellea*, but with longer periods substrate value for *A. mellea* progressively declined. Segments of living, green shoots of willow provided a consistently better substrate for *A. mellea* than did dead segments, and these living segments maintained their substrate value over periods (up to 7 weeks) of previous burial in soil. These results are interpreted in terms of competition between *A. mellea* and other soil fungi. In the infection of living tissues, *A. mellea* benefits from the exclusion of competitors by host resistance. In the saprophytic colonization of dead tissues, *A. mellea* has the advantage of its capacity to decompose cellulose and lignin, which are substrates restricted to a minority of soil fungi.

INTRODUCTION

THE saprophytic behaviour of most root-infecting fungi is still a matter for speculation. The laboratory experiments so far carried out represent a first, crude approximation to a whole series of complex ecological situations in the field. Various tests are available for determining presence/absence of a particular root-infecting fungus in a substrate unit of plant tissue, exposed to saprophytic colonization by the fungus in competition with other soil micro-organisms under standardized conditions (Garrett, 1956*a*, p. 141). The percentage of such substrate units colonized by the fungus under investigation has been used as a measure of its *competitive saprophytic ability* in the particular situation represented by the test. This procedure gives no information about the actual *proportion* of plant tissue that is colonized and exploited by the fungus in all or any of the substrate units; the tests indicate only presence/absence.

No method has yet been developed for determining at all precisely the proportion of a substrate colonized by a fungus in the presence of and in competition with other fungi and other soil micro-organisms. This difficulty does not arise in the much simpler situation presented by a pure culture; here the progress of colonization can be continuously followed by measurements of

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

loss in dry weight or of carbon dioxide respired, or by chemical determinations of rate of change in selected constituents of the substrate.

For the investigation of this particular problem, *A. mellea* offers one advantage at present unique amongst the root-infecting fungi that have been sufficiently studied as experimental subjects. If a corpus of plant tissue that has been colonized by *A. mellea* is buried in soil, it eventually produces rhizomorphs; the rate and duration of rhizomorph growth appear to be related to the volume of *A. mellea* mycelium in the corpus of tissue. In the preceding paper of this series (Garrett, 1956*b*), I reported a method for measuring weekly-growth increments of rhizomorphs put out from woody inoculum segments in glass tubes filled with soil. Mean weekly-growth increments of rhizomorphs over the first 5 weeks could be correlated with volume of *A. mellea* mycelium in the inoculum segment serving as the food base; as these experiments were carried out with inoculum segments colonized by *A. mellea* in pure culture, the volume of *A. mellea* mycelium could be directly related to volume of the woody inoculum segment. To establish more precisely the quantitative relationship between volume of *A. mellea* mycelium in the inoculum segment and mean weekly-growth increments of the rhizomorphs put out from it will require more elaborate experiments. This further calibration of the technique has been postponed until its usefulness could be assessed by a preliminary trial on several different problems. Thus I have used it to obtain an estimate of loss of viability by *A. mellea* in woody inoculum segments exposed to carbon disulphide or to the antibiotic activity of *Trichoderma viride* (Garrett, 1957, 1958). The present paper reports its preliminary application to quite a different type of problem.

Interest in the practical question of the extent to which *A. mellea* can colonize dead woody substrates in competition with other soil fungi was aroused by Leach (1937, 1939) in two papers from Nyasaland that have profoundly influenced subsequent work on root disease problems in tropical crops. Leach found that when forest was cleared by felling in preparing the site for a tea plantation, the extent of infection of the stump roots of the former forest trees could be correlated with length of time for which these roots remained alive after felling of the trees. This conclusion was supported by experiments with ring-barking as a method for rapid killing of the root systems, through depletion of their starch reserves. Leach therefore concluded that once tree roots were killed and then occupied by saprophytic fungi, *A. mellea* was no longer able to invade them. A field experiment by Wiehe (1952) subsequently provided substantial evidence for the practical value of the ring-barking method as an adjunct to forest clearing in preparation for a plantation crop.

Leach's conclusions seem so credible that the need for further investigation could be questioned. Nevertheless, he did not determine the precise extent of colonization by saprophytic fungi that was required to prevent the entry and development of *A. mellea* in dead tree roots, and there is scope for further investigation here. It is not inconsistent with Leach's conclusions to suppose that the rhizomorphs of *A. mellea* may have quite a high degree of competitive saprophytic ability, and may be able to colonize dead roots in competition with saprophytic fungi unless and until the roots have been completely occupied by earlier arrivals. Even for a root-infecting fungus that is able to colonize dead plant tissues in competition with saprophytes, living host roots provide a better substrate than do dead ones; in living roots, host resistance excludes the entry of all micro-organisms except parasites, and so a parasite can exploit the living tissues at first virtually in pure culture. At present there is suggestive though not conclusive evidence that the rhizomorphs of *A. mellea* can sometimes colonize dead wood saprophytically, and Cartwright and Findlay (1958, p. 73) have reported the not infrequent occurrence of rhizomorphs on pit props in damp coal-mines. It is conceivably possible that all such occurrences of *A. mellea* can be ascribed to earlier infection of the pit prop, whilst it was still a living young tree, and not to saprophytic colonization of the dead pit prop at a later stage in the coal-mine. In order to provide evidence bearing on this and similar questions, the experiments now to be described were undertaken.

EXPERIMENTAL WORK

The procedure for producing pure cultures of *A. mellea* in woody inoculum segments has been described in preceding papers (Garrett, 1956*b*, 1957). So has the method for setting up inoculum segments in glass tubes of moist soil, and for measuring rhizomorph growth in either direction from the inoculum segment, whereby mean weekly-growth increments can be calculated.

The general procedure for these experiments was as follows. Woody substrate segments cut from shoots of cricket-bat willow (*Salix alba* var. *coerulea*) were killed by autoclaving (to destroy host resistance to invasion) and then buried in soil for various periods, to permit of colonization by soil fungi and other organisms. After its period of incubation in soil each substrate segment was set up individually in a glass inoculation tube, in which it was separated from a woody inoculum segment (permeated by a pure culture of *A. mellea*) by a 2 cm. column of soil. Contact with a soil column induces the inoculum segment to produce rhizomorphs, which are not usually initiated when the inoculum segment is buried in moist sand (Garrett, 1956*b*). Substrate segments were incubated in these tubes along with their inoculum segments for 4 weeks at 22° C., to give opportunity for rhizomorphs from the inoculum segment to colonize the substrate segment. After this incubation period all substrate segments were removed from the inoculation tubes and set up in growth tubes of fresh soil, held at 25° C., for measurement of rhizomorph growth-rate. Mean weekly-growth increments of rhizomorphs over the first 5 weeks of growth from each substrate segment were then calculated, to give an estimate of the extent to which the substrate segment had been colonized by *A. mellea* in the inoculation tube.

Experiment 1. The objectives of this experiment were (1) to compare segments of living, green willow shoots with similar segments killed by autoclaving, as substrates for *A. mellea* after a previous period of burial in soil, (2) to determine the effect of time in soil (resulting in progressive colonization of dead segments by soil saprophytes) on value of the segments as substrates for *A. mellea*. Substrate segments were prepared in the first place as 12·5 cm. lengths cut from shoots of cricket-bat willow (*Salix alba* var. *coerulea*) not exceeding 1·75 cm. diameter; the fresh weights of all segments collected for this experiment were within the range 21–26 g. At each collection half these long segments were autoclaved for 30 min. at 1 atm. over moist sand. Both fresh and autoclaved segments were then buried in 2 lb. glass jam-jars of soil held at a moisture content of 60 per cent. m.h.c.; half were in Kettering soil and the other half in garden soil. The Kettering soil is a reddish loam, collected from under old pasture, high in organic matter and total nitrogen but low in available phosphate and potash, with pH value ranging from 5 to 6. The garden soil is a greyish, light loam, fairly high in organic content, from my own garden at Cambridge, with pH value around 6·5. Collection, preparation, and burial in soil of serial batches of these long substrate segments was carried out at periods of 28, 14, 7, and 0 days before the beginning of the second stage of the experiment. The soil jars were incubated at laboratory temperature (mean, 21° C.; range, 18–24° C.).

For the second stage of the experiment, the various batches of long substrate segments were removed from the soil jars and brushed clean; the 0-day series of segments were freshly prepared and had not been buried in soil at all. From the bottom end of each of these long segments, three short segments of length 2·5 cm. were sawn off successively, and numbered 1, 2, 3 from the bottom end upwards. Each short substrate segment was then placed individually in an inoculation tube (25 cm. long × 1·9 cm. internal diameter), containing an inoculum segment in contact with a 2 cm. column of garden soil at a moisture content of 60 per cent. m.h.c. (Fig. 1). Inoculum segments had previously been prepared from 2·5 cm. lengths of willow shoot not exceeding 1·75 cm. diameter, and their mean fresh weight was 4·25 g. After autoclaving and inoculation with *A. mellea* they had been incubated at 25° C. for 2½ months. Inoculation tubes had been set up, with inoculum segment and short soil column, 13 days before substrate segments were due to be introduced; previous experience had shown that 13 days at 25° C. was rather less than the minimum time required for rhizomorphs to be initiated and then to grow through a 2 cm. column of soil. After the substrate segment had been placed in the inoculation tube at that end of the soil column remote from the inoculum segment (Fig. 1), a 5 cm. column of sand was added and brought to a moisture content of 100 per cent. m.h.c. This column of moist sand, like the similar column at the other end of the tube beyond the inoculum segment, served to keep the segment in place and to prevent drying-out. The completed inoculation tubes were capped with lacquered, moisture-proof 'Cellophane' and incubated at 22° C. for 4 weeks.

In a majority of the inoculation tubes, a previous incubation period of 13 days at 25° C. had been insufficient for rhizomorphs to be initiated and to grow through the 2 cm. soil column. Those inoculation tubes in which rhizomorphs had not yet made contact with the substrate segment were therefore inspected at daily intervals and the date on which contact was finally established was noted. The additional number of days was then added to the previous incubation period of the substrate segment in a soil jar.

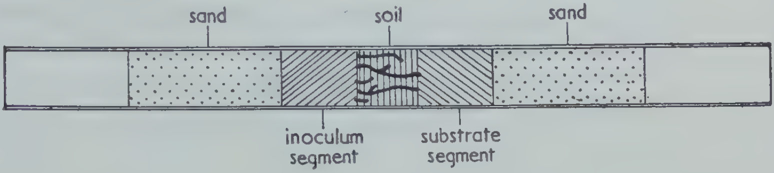

FIG. 1. Inoculation tube (× 10/25).

For the third stage of the experiment, substrate segments were removed from their inoculation tubes and brushed clean of soil. Each substrate segment was then set up at the mid-point of a rhizomorph growth-tube (25 cm. long × 1·9 cm. internal diameter), filled with garden soil at a moisture content of 60 per cent. m.h.c. Rhizomorph growth-tubes were incubated at 25° C. (optimum for rhizomorph growth) for weekly-growth recording.

Before setting out the results obtained from this experiment the numerical basis for comparison of each series must be given. At 28, 14, 7, and 0 days before setting up the second stage, in the inoculation tubes, two living and two autoclaved long segments had been buried in each of the two soils, Kettering and garden. This yielded six living and six autoclaved short segments from each soil for setting up as substrate segments in the inoculation tubes. A preliminary assessment of results showed that neither the type of soil (Kettering *v.* garden) nor the position (1, 2, 3 from the bottom upwards) of short segments on the original long segments had significantly affected the results. The final analysis of the data was therefore restricted to the following comparisons: (1) living *v.* dead substrate segments, (2) time of previous burial in soil before rhizomorphs from the inoculum segment had made contact with the substrate segment. After adding the additional incubation periods in the inoculation tubes to the original incubation period of the long segments in soil jars, the following series of incubation periods were obtained: 0–7, 14, 21, 28, and 35–49 days. In each case the incubation period was calculated to the nearest week, and data for 0–7 days, and again for 35–49 days, were grouped together so as to secure a sufficient number of replicates for each group. The mean weekly-growth increments of rhizomorphs given in Table 1 are calculated from measurements of rhizomorph growth from both ends of all substrate segments in each series, including those segments from which no rhizomorphs were produced.

In interpreting the data given in Table 1, one fundamental assumption has to be made—that the mean weekly growth increment of rhizomorphs over the first 5 weeks, as given in the final column, is related to the volume of *A. mellea* mycelium in the substrate segment. Granted this assumption, the evidence for which has been presented in the Introduction, the following conclusions can be drawn. Firstly, living substrate segments, which are *infected* by the fungus, have provided a consistently better substrate than have dead segments, which are *saprophytically colonized.* The mean weekly-rhizomorph-growth increment over the whole experiment is 14 for living segments and 9 for dead ones. For living segments there is no decline in value as a substrate for *A. mellea* after 28 days' previous burial in soil; the slight decline shown when the previous burial period exceeded 28 days is not significant ($P = 0\cdot1$).

TABLE 1

Successive Weekly-Growth Increments of Rhizomorphs (mm.), as an Estimate of Substrate Colonization

	Weekly periods					Mean
	1	2	3	4	5	(±1·10)
Living substrate segments						
0–7 days	24	14	16	8	9	14
14 days	18	15	9	18	11	14
21 days	17	14	11	15	16	15
28 days	22	16	16	12	11	15
35–49 days	20	11	10	13	5	12
Dead substrate segments						
0–7 days	12	8	6	6	3	7
14 days	17	9	10	9	9	11
21 days	17	10	14	9	10	12
28 days	15	7	8	10	7	9
35–49 days	9	2	5	2	4	4

Standard error for individual weekly means = 2·46

For dead segments a period of previous burial in soil not exceeding 21 days, during which the segments were progressively invaded by saprophytes, did not result in any decline in substrate value. Indeed, the figure for 0–7 days' previous burial is significantly *lower* ($P = 0\cdot02$) than those for the longer periods of 14 and 21 days—an anomalous result for which no explanation can be given. After a previous burial period of more than 21 days there was a progressive and significant decline ($P < 0\cdot001$) in the substrate value of dead segments, from a value of 12 at 21 days to one of 4 at 35–49 days.

One further comment is required about the figures given in Table 1. In my previous paper (Garrett, 1956*b*) weekly-growth increments of rhizomorphs showed a steady downward trend from the first to the fifth weekly period. In Table 1 here this downward trend is not so regular. This difference can probably be related to the fact that the previous experiments were carried out with inoculum segments thoroughly permeated by *A. mellea* in pure culture, whereas in the present experiments the inoculum segments were colonized in competition with other organisms, and the distribution of *A. mellea* mycelium must have been much more irregular.

Such dead segments of bark-covered wood are rapidly invaded by fungi and other saprophytes when buried in soil, as many previous workers have shown, and no useful purpose would have been served by attempting isolation and identification of even the fungal invaders prior to *A. mellea*, especially as the preliminary assessment of results had revealed no significant effect of soil type on later colonization by *A. mellea* (see above). A more significant estimate of the effect of burial of the long segments in the soil jars was obtained by noting the degree of visible decomposition of the bark. For this purpose, the living segments acted as a control; their bark remained firm and green up to the end of the 28-day period in the soil jars; all segments had produced roots and some had produced one or more shoots as well. In contrast the bark of the dead segments had become very soft after only 7 days in soil, and this deterioration in the condition of the bark increased with longer burial. Incidentally, it can be noted that progressive invasion of the living segments by rhizomorphs in the inoculation tubes could be followed through change in colour of the bark from green to brown.

Experiment 2. The objectives of this experiment, which was carried out with dead substrate segments only, were: (1) to test the conclusions from Expt. 1, with respect to period of previous burial in soil as affecting substrate availability for *A. mellea*, (2) to determine whether variation in the inoculum potential of rhizomorphs would affect the degree of substrate colonization. The inoculum potential of rhizomorphs was reduced in two ways: (1) by decreasing the size of inoculum segments, (2) by increasing the distance between inoculum and substrate segments. Three types of inoculation tube were therefore set up, (1) with inoculum segment of mean fresh weight 4·25 g., separated from substrate segment by 10 cm. column of soil, (2) with inoculum segment of mean fresh weight 4·25 g. and (3) of 1.29 g., in each series separated from substrate segment by 2 cm. soil column. Preliminary assessment of the results showed no significant differences arising from these different types of inoculation, and so the factorial experiment has been analysed and presented as a simple randomized block experiment on period of previous burial of substrate segments in soil.

In other respects, the general procedure for setting-up this experiment was similar to that for Expt. 1, but with the following differences in detail. Firstly, the substrate segments were sawn into their ultimate lengths of 2·5 cm. (mean fresh weight 4·5 g.) before being autoclaved for 30 min. at 1 atm. over moist sand. Secondly, all of them were then incubated for 14 days in jars of garden soil at a moisture content of 60 per cent. m.h.c. (laboratory temperature 20–22° C.). At the end of this period, substrate segments were transferred individually to inoculation tubes with garden soil at 60 per cent. m.h.c. separating inoculum and substrate segments, and held at 22° C. for 4 weeks. The further period elapsing before rhizomorphs made contact with the substrate segment in each inoculation tube was added to the previous incubation period of 14 days in the soil jars, and so substrate segments could be grouped for total incubation periods in soil of 14, 21, 28, and 35 days (calculated to the nearest week).

Fifty-seven substrate segments were available for the third and final stage of the experiment, in rhizomorph growth-tubes. Any substrate segment that rhizomorphs had failed to reach less than one full week before the end of the incubation period in the inoculation tubes had been discarded at the end of the second stage. One week's contact with the growing rhizomorphs was deemed, from actual observation of events through the glass wall of the inoculation tube, to be sufficient for saprophytic colonization to be initiated and to progress. Substrate segments colonized later than others in the inoculation tubes might have been expected to show a comparable delay in initiation of rhizomorphs in the growth-tubes during the third stage of the experiment. In the event, mean time to first initiation of rhizomorphs by substrate segments in the growth-tubes showed relatively small differences between the various series, as follows: 14-day series, 2·28 weeks; 21-day series, 2·21 weeks; 28-day series, 2·44 weeks; 35-day series, 3·0 weeks. Mean weekly-growth increments of rhizomorphs given in Table 2 are calculated from measurements of rhizomorph growth made from both ends of all substrate segments, including those segments from which no rhizomorphs were produced.

TABLE 2

Successive Weekly-Growth Increments of Rhizomorphs (mm.), as an Estimate of Substrate Colonization

	Weekly periods					Mean
	1	2	3	4	5	(±0·89)
14 days	19	9	14	9	13	13
21 days	18	11	12	10	10	12
28 days	16	5	7	8	10	9
35 days	4	1	2	1	3	2

Standard error for individual weekly means = 1·99

The substrate colonization values for *A. mellea*, as expressed in Table 2 by mean weekly-growth increments of rhizomorphs, compare very closely with those given for corresponding series of dead substrate segments in Table 1. As in Expt. 1, a period of up to 21 days' burial in soil preceding actual contact with the colonizing rhizomorphs has not reduced substrate value for *A. mellea*. Once again, however, periods of 28 and 35 days' previous burial in soil have rendered substrate segments progressively less available for *A. mellea*. The decline in colonization value from 12 (for 21 days) to 9 (for 28 days) is significant at the 2 per cent. level, and the sharper decline to 2 (for 35 days) is significant at the 0·1 per cent. level.

Experiment 3. The results obtained in Expts. 1 and 2 would be difficult to understand if *A. mellea* were able to utilize only sugars and the simpler carbon compounds, because these constituents are the first to be decomposed and used up when a woody substrate is invaded by fungi and other soil micro-organisms. There is little doubt, however, that *A. mellea* can decompose both cellulose and lignin. The fungus causes a white rot of the wood, typical of fungi that decompose lignin as well as cellulose (Cartwright and Findlay, 1958), and the woody cylinder of an infected root is eventually reduced to a mere sponge, from which water can be squeezed out. Although the ability of *A. mellea* to decompose both cellulose and lignin is scarcely open to question, it was nevertheless of interest to test the efficiency in cellulose decomposition of the actual isolate of the fungus (originally obtained from an apple root) used in these experiments. Whatman No. 3 Filter Paper (extra thick: ash weight 0·001 g.) of 7 cm. diameter was made up in wads of ten papers, which were moistened in 250 ml. conical flasks with 20 ml. nutrient solution of the following composition: peptone (13·2% N), 12·3 g.; K_2HPO_4, 2·0 g.; $MgSO_4.7H_2O$, 1·0 g.; $FeCl_3$, 0·2 mg.; thiamin, 0·1 mg.; distilled water, 1 litre. After autoclaving for 30 min. at 1 atm. fourteen flasks were inoculated with 12·5 mm. discs cut marginally from a growing colony of *A. mellea* on 3 per cent. malt extract agar, and six flasks were left uninoculated as controls. Flasks were thereafter incubated in the dark at a mean laboratory temperature of 20° C.

After a slow start, the colonies of *A. mellea* grew vigorously on the wads of moist filter-paper, and produced abundant rhizomorphs interleaving the papers. After 16 weeks' growth both cultures and uninoculated controls were removed from the flasks and dried to constant weight at 80° C. Mean percentage loss in dry weight of the inoculated wads of filter-paper was $9·5 \pm 0·17$. This loss represents only that proportion of the cellulose actually respired by *A. mellea*, and does not include the proportion converted into fungal substance.

DISCUSSION

The results reported above necessitate some revision of ideas concerning the saprophytic behaviour of root-infecting fungi. *Armillaria mellea* has some, at least, of the attributes of a highly specialized root parasite. It has a capacity for potentially unlimited spread over the root systems of its woody host plants, which bespeaks a high degree of efficiency in infection. Advance of the fungus is sometimes along the plane of weakness between bark and wood, and sometimes it is ectotrophic; the ectotrophic root-infecting habit is characteristic of highly specialized parasites and I have pointed out that it is the manifestation of a capacity for potentially unlimited spread over the host root system (Garrett, 1956*a*, ch. 4). In one respect, however, the behaviour of the rhizomorphs of *A. mellea* shows a marked difference from that of rhizomorphs of other specialized parasites of tree roots. In most of such fungi, rhizomorphs and mycelial strands are not found growing through the soil apart from root systems, though sometimes they will cover a *continuous* solid surface provided by boulders and large stones, or will grow along dead roots. The rhizomorphs of *A. mellea* are less closely associated with root systems, inasmuch as they will grow out freely from a food base through soil quite empty of roots, living or dead.

Yet another criterion of parasitic specialization, albeit a negative one, has been argued from saprophytic behaviour (Garrett, 1950, 1956*a*). I have classified root-infecting fungi into two broad ecological groups: the primitive parasites, or *soil-inhabiting fungi*, capable of living as competitive saprophytes in the soil, and the specialized parasites, or *root-inhabiting fungi*, incapable of free saprophytic existence, though able to survive saprophytically in dead host tissues infected during the parasitic phase. There were good arguments for supposing that a high degree of competitive saprophytic ability was incompatible with evolution of the host-parasite relationship beyond a certain point, and so the low saprophytic ability found in some specialized parasites seemed readily explicable.

This distinction still seems to be reasonably serviceable for fungi infecting the roots of herbaceous plants. But there are now indications that the behaviour of some fungi infecting tree roots may be so different as to make this distinction sometimes quite unreal. Thus the results reported in this paper show that *A. mellea*, which has many of the attributes of a specialized parasite, can colonize dead woody tissues lying in the soil at least for some weeks after they have been opened to colonization by other fungi.

This apparent contradiction within our admittedly limited experience may be due to a fundamental difference in the type of substrate offered by the dead roots of herbaceous plants and of woody plants,. A relatively high proportion of tissue constituents in dead roots of herbaceous plants is available for exploitation by the great majority of soil fungi; competition for saprophytic colonization is accordingly very intense, and even a slight handicap imposed by specialization to a parasitic life may be sufficient to exclude a parasite from success as a competitive saprophyte on such substrates. For success in saprophytic colonization of woody tissues, on the other hand, the ability to decompose cellulose (±lignin) is probably the most essential qualification. Strong cellulose decomposers are in a numerical minority amongst species of soil fungi, and strong lignin decomposers in an even smaller minority. Saprophytic competition amongst this élite may be no less intense when issue is joined, but encounters must be less frequent and so such a fungus has a greater chance of being a pioneer saprophytic colonizer in so far as competitors of its own kind are concerned. In such a situation, a handicap imposed by some degree of specialization to a parasitic life may be less of a disadvantage, if only because a fungus like *A. mellea* has an appreciable chance of being the first cellulose and lignin decomposer to arrive at a virgin woody substrate—an event that may well be rendered more frequent by the habit of free rhizomorph growth through the soil, outwards in all directions from a food base.

This argument may be stated in more general terms by saying that if a fungal species has acquired, through evolution, the ability to colonize a type of substrate not accessible to the majority of other fungal species, it then escapes into an area of reduced competition. In the same way some species of higher plants have exchanged competition pressure for a more rigorous environment, which in the extreme case arrests plant succession in the stage of an open community, with bare ground between individual plants. Cellulose is a substrate not accessible to a majority of soil fungi; lignin is still less accessible, and so these substrates remain open to exploitation for much longer than do simpler substrates. The root systems of higher plants similarly furnish a waiting substrate for the small number of parasites that can invade them without effective check by host resistance. Some fungi that are specialized parasites of tree roots can decompose both cellulose and lignin; in their parasitic phase they virtually escape competition altogether, and in their saprophytic phase the intensity of competition to which they are exposed is much reduced by their special biochemical abilities in substrate decomposition.

ACKNOWLEDGEMENT

I am much indebted to Dr. J. Rishbeth for discussion of the manuscript.

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Note on a Rapid Method for Wax Embedding

BY

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THE conventional method of preparing plant material for microtoming by fixing, dehydrating, and embedding in wax occupies several days. The modified process described below reduces the time required to 24 hours. This method is particularly suitable for buds, where the soft inner tissues are enclosed in brittle scales.

The essential features are the use of a glycerine-methylated spirits mixture after fixing to soften the brittle tissues, and the employment of reduced air pressure to minimize the time of treatment with alcohols and cedarwood oil, both of which have a hardening effect. The materials, in suitable containers, can conveniently be exposed to partial evacuation (approx. 100 mm. mercury residual pressure) in a vacuum desiccator chamber from which the desiccant has been removed. Too low a pressure or too long an exposure may cause collapse of delicate structures, so the technique is not suitable for very fragile tissues.

The recommended procedure is as follows:

(1) Fix under reduced pressure for approx. 15 min. in formo-acetic acid mixture (formalin 5, glacial acetic acid 5, 50 per cent. alcohol 90 parts).

(2) Leave 3–12 hrs. in equal parts of glycerine and methylated spirits.

(3) Rinse 1–3 hrs. in two changes of distilled water at 45° C.

(4) Take up through the alcohols and cedarwood oil at reduced pressure, as follows:

50% alcohol .	15 min.
70% alcohol .	10 min.
95% alcohol .	5 min.
absolute alcohol .	5 min.
cedarwood oil .	15 min.

(5) Embed in paraffin wax (m.p. 55°) kept at 65° – 70° C. by standing container in hot water. (This stage must be carefully watched, so that normal air pressure can be quickly restored as soon as the wax appears likely to overflow.)

(6) Change into clean wax, leave in embedding oven at 60° C. for 12 hrs., then prepare block for microtoming.

[Annals of Botany, N.S. Vol. 24, No. 94, 1960.]

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